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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
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NOVEL TARGETS TO IMPROVE AXONAL REGENERATION IN THE CNS:

THE ROLE OF MYELIN LIPID INHIBITORS, INJURY SIGNALS AND AXONAL TRANSPORT.

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TESE DE DOUTORAMENTO APRESENTADA

AO INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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NOVEL TARGETS TO IMPROVE AXONAL REGENERATION IN THE CNS: THE ROLE OF MYELIN LIPID INHIBITORS, INJURY SIGNALS AND AXONAL TRANSPORT.

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“Mais vale ser que parecer”

(Provérbio popular português)

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Summary

In the adult central nervous system (CNS), injured axons have limited ability to regenerate. This regenerative failure is accounted in part by the fact that upon lesion, an inhibitory glial scar is formed. Besides, injured CNS neurons are not able to mount a regenerative program and express the genes needed to promote axonal elongation. In contrast, in the peripheral nervous system (PNS), axons are able to regenerate given the efficient Wallerian degeneration, the removal of inhibitory molecules by Schwann cells and macrophages, and the ability to express regeneration-associated genes as a response to the retrograde transport of injury signals.

In this Thesis we aimed at further dissecting mechanisms related to the failure of axonal regeneration in the CNS namely: i) the inhibitory action of different myelin components; ii) the retrograde transport of injury signals and iii) the anterograde transport of regeneration enhancers.

Most of the inhibitory molecules identified following CNS injury are myelin-associated proteins and chondroitin sulphate proteoglycans. By using shiverer mice, we aimed at further unraveling the importance of myelin during axonal regeneration, as this model lacks compact myelin in the CNS. Although shiverer mice displayed a standard glial scar, an increased axonal regeneration and sprouting was observed after spinal cord injury. *In vitro*, we demonstrated that besides myelin proteins, myelin lipids, that are severely decreased in the shiverer spinal cord, specifically cholesterol and sphingomyelin, were inhibitory for axon outgrowth through a mechanism involving Rho activation. *In vivo*, and supporting the importance of myelin lipids in repressing axonal regeneration, 2-hydroxypropyl-β-cyclodextrin, a drug that reduced lipid levels in the injury site, promoted axonal regeneration following spinal cord injury. In summary, our work supports that myelin lipids should be considered together with myelin proteins as targets to improve axonal growth following injury.

ERK, JNK and STAT-3 are positive injury signals that trigger a regenerative response following PNS lesion. To further understand the importance of injury signaling, we used dorsal root ganglia (DRG) neurons that comprise a central branch that does not regenerate and a peripheral branch that regrows after lesion. Whereas injury to the central branch of DRG neurons also led to activation and retrograde transport of ERK, JNK and STAT-3, only injury to the peripheral branch was able to elicit a gain in intrinsic growth capacity. By analysis of dynein-

bound axoplasm proteins through antibody microarrays following either peripheral or central branch injury, a broad differential activation and transport of signals after each injury type was observed. From these, increased levels of Hsp-40, ROCK-II and GSK3β after central branch injury were identified not only in the axoplasm but also in DRG cell bodies. In summary, activation and transport of canonical positive injury signals is not sufficient to increase intrinsic growth capacity, and limited regenerative response may be accounted by activation of inhibitory injury signals including ROCKII and GSK3β.

To study the anterograde transport of regeneration enhancers, *in vivo* radiolabeling of DRG neurons coupled to mass spectrometry and kinesin immunoprecipitation of spinal cord extracts was performed. Following peripheral conditioning lesion, increased intrinsic growth capacity was accompanied by increased anterograde transport of cytoskeleton components, metabolic enzymes and potential axonal regeneration enhancers, in the central branch of DRG neurons. Changes in axonal transport induced by peripheral conditioning were broad including mitochondria, lysosomes and synaptic vesicles. In summary, a peripheral injury induces a global increase in axonal transport that by extending to the central branch, allows a rapid and sustained support of regenerating central axons.

Overall, in this Thesis we contributed to: i) characterize cholesterol and sphingomyelin as novel axonal regeneration inhibitors; ii) identify ROCK-II and GSK3β as repressors of axonal regeneration that are linked to the retrograde transport machinery and iii) identify augmented axonal transport as a key feature of the increased regeneration ability produced after a peripheral conditioning injury.

Sumário

No sistema nervoso central (SNC), após lesão os axónios têm pouca capacidade de regeneração. Este facto deve-se à formação de uma cicatriz glial inibitória após lesão. Além disso, os neurónios do SNC não são capazes de produzir uma resposta regenerativa e expressar os genes necessário à regeneração quando lesionados. Pelo contrário, no sistema nervoso periférico (SNP), os axónios são capazes de regenerar devido a uma eficiente degeneração Walleriana onde há a remoção de moléculas inibitórias pelas células de Schwann e macrófagos, e devido ainda à sua capacidade para expressar genes associados à regeneração como resposta ao transporte retrogrado de sinais positivos de regeneração.

Nesta Tese, os nossos objectivos foram desvendar mecanismos relacionados com a ausência de regeneração após lesão no SNC nomeadamente: i) a acção inibitória de diferentes componentes da mielina; ii) o transporte retrogrado de sinais de regeneração e iii) o transporte anterogrado de potenciadores de regeneração.

A maior parte das moléculas inibitórias identificadas após lesão no SNC são proteínas associadas à mielina e proteoglicanos de sulfato de condroítina. Usando ratinhos shiverer procuramos perceber a importância da mielina na regeneração axonal, uma vez que este modelo não apresenta mielina compacta no SNC. Apesar de os ratinhos shiverer formarem uma cicatriz glial semelhante a ratinhos selvagens, observamos que os seus axónios têm maior capacidade de regeneração e maior plasticidade após uma lesão na espinal medula. *In vitro*, mostrámos que além das proteínas da mielina, os seus lípidos que estão bastante diminuídos na espinal medula de ratinhos shiverer, especificamente colesterol e esfingomielina, são inibitórios para o crescimento axonal por um mecanismo dependente na activação de Rho. Suportando o facto de os lípidos da mielina serem inibidores da regeneração axonal, o uso *in vivo* de 2-hidroxipropil-β-ciclodextrina, um fármaco capaz de reduzir a quantidade de lípidos acumulada na zona lesionada, aumentou a regeneração axonal após lesão na espinal medula. Em suma, o nosso trabalho mostra que além das proteínas da mielina, os seus lípidos também deveriam ser considerados alvos para promover regeneração axonal após lesão.

ERK, JNK e STAT-3 são sinais positivos de regeneração que são activados após lesão no SNP e desencadeiam uma resposta regenerativa. Para melhor compreender a importância dos sinais de regeneração, usámos neurónios dos

gânglios da raiz dorsal que possuem um ramo central que não é capaz de regenerar e um ramo periférico que regenera quando lesionado. Apesar de uma lesão no ramo central dos neurónios dos gânglios da raiz dorsal também levar à activação e transporte retrogrado de ERK, JNK e STAT-3, apenas a lesão no ramo periférico é capaz de promover um aumento na capacidade regenerativa desses neurónios. A análise através de um microarray de anticorpos das proteínas axoplasmáticas ligadas à dineína após lesão no ramo periférico ou central permitiu a identificação de alterações extensas na activação e transporte de sinais de lesão. Destes, podemos destacar um aumento de Hsp-40, ROCK-II e GSK3β após lesão no ramo central, não apenas no axoplasma, mas também no corpo celular dos neurónios dos gânglios da raiz dorsal. Em suma, a activação e transporte de sinais positivos de lesão não é suficiente para aumentar a capacidade intrínseca de regeneração dos neurónios. A baixa capacidade regenerativa poderá também ser devida à activação de sinais de lesão inibitórios como ROCK-II e GSK3β.

Para estudar o transporte anterogrado de potenciadores de regeneração, os neurónios dos gânglios da raiz dorsal foram marcados com radioactividade *in vivo* e posteriormente amostras de espinal medula foram analisadas por espectrometria de massa e imunoprecipitação de quinesina. Após lesão periférica condicionante, o aumento na capacidade intrínseca de regeneração foi acompanhada por um aumento do transporte anterogrado de componentes do citoesqueleto, enzimas metabólicas e possíveis potenciadores de regeneração no ramo central dos neurónios dos gânglios da raiz dorsal. As alterações no transporte axonal despoletadas por uma lesão periférica condicionante foram bastante extensas e incluem aumento no transporte de mitocôndrias, lisossomas e vesiculas sinápticas. Em suma, uma lesão periférica condicionante induz um aumento global no transporte axonal no ramo central dos neurónios dos gânglios da raiz dorsal, que permitem uma resposta regenerativa rápida e continuada.

Globalmente, nesta tese nós contribuímos para: i) a caracterização o colesterol e esfingomielina como novos inibidores de regeneração; ii) a identificação de ROCK-II e GSK3β como estando ligados à maquinaria de transporte retrogrado levando à repressão da regeneração axonal e iii) a identificação do aumento do transporte axonal como uma característica fundamental para o aumento da capacidade regenerativa produzida por uma lesão condicionante periférica.

Abbreviation list

5HT	5-hydroxytyptamine
9CPA	9-cyclopentyladenine
aCSF	Artificial CSF
ALS	Amyotrophic lateral sclerosis
AKT	Protein kinase B
APC	Anaphase promoting complex
Arg-1	Arginase 1
ATF-3	Activating transcription factor 3
ATP	Adenosine-5'-triphosphate
BBB	Blood-brain-barrier
BDNF	Brain-derived neurotrophic factor
C3	C3-ADP-ribosyltransferase
cAMP	Cyclic adenosine monophosphate
CE	Cholesteryl esters
Cer	Ceramide
cGMP	Cyclic guanosine monophosphate
CGN	Cerebellar granule neurons
CNS	Central nervous system
CO	Cholesterol
CREB	cAMP response element-binding protein
CRMP-2	Collapsin response mediator protein 2
Csk	C-terminal Src kinase
CSPG	Chondroitin sulfate proteoglycans

CST	Corticospinal tract
CTB	Cholera toxin B
db-cAMP	Dibutyryl cyclic adenosine monophosphate
DLK	Dual leucine zipper kinase
DREZ	Dorsal root entry zone
DRG	Dorsal root ganglia
DRI	Dorsal root injury
ECM	Extracellular matrix
EGL	External plexiform layer
Elk-1	E twenty-six like transcription factor 1
ERK	Extracellular signal regulated kinase
ESCs	Embryonic stem cells
GalCer	Galactocerebroside
GAP-43	Growth associated protein 43
Gb4	Globotetrahexosylceramide
GDNF	Glial-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GM1	Ganglioside GM1
GS	Sulfatide
GSK3β	Glycogen synthase kinase 3 beta
HPβCD	2-hydroxypropyl-β-cyclodextrin
HDAC5	Histone deacetylase-5
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HPTLC	High-performance thin-layer chromatography

IGL	Inner granule layer
IL-1α	Interleukin-1α
IL-6	Interleukin-6
IL-10	Interleukin-10
JNK	c-Jun N-terminal kinases
Lac	Lactocerebroside
LIF	Leukemia inhibitory factor
MAG	Myelin associated glycoprotein
MAIs	Myelin associated inhibitors
MAP1b	Microtubule-associated protein 1B
MAPKKK	Mitogen-activated protein kinase kinase kinase
MBP	Myelin basic protein
MCP-1	Monocyte chemoattractant protein-1
MHC-II	Major histocompatibility complex II
mRNA	Messenger RNA
MSC	Bone marrow stromal cells
mTOR	Mammalian target of rapamycin
NCAM	Neural cell adhesion molecule
NFIL3	Nuclear factor, interleukin 3 regulated
NGF	Nerve growth factor
NgR	Nogo-66 receptor
NLS	Nuclear localization signal
Npc1	Niemann-Pick type C1
NPY	Neuropeptide Y

NSCs	Neural stem cells
NT-3	Neurotrophin 3
NT-4	Neurotrophin 4
OEC	Olfactory ensheating cells
OMgp	Oligodendrocyte myelin glycoprotein
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PI3K	Phosphoinositide 3-kinase
Plk3	Polo-like kinase 3
PNS	Peripheral nervous system
PS	Phosphatidylserine
PTEN	Phosphatase and tensin homologue
RAGs	Regeneration associated genes
RGM	Repulsive Guidance Molecules
RhoGDI	Rho GDP dissociation inhibitor
ROCK	Rho-associated kinase
SEMA-3	Class 3 semaphorins
Shi	Shiverer
Smad1	Mothers against decapentaplegic homolog 1
SOCS-3	Suppressor of cytokine signaling 3
Sox11	sex determining region Y-box 11
SCa	Slow component a
SCb	Slow component b

SCI	Spinal cord injury
SNI	Sciatic nerve injury
SpH	Sphingomyelin
Src	Proto-oncogenic cytoplasmic tyrosine kinase
STAT-3	Signal transducer and activator of transcription 3
Syd	Sunday Driver
TG	Triglycerides
TNF-α	Tumor necrosis factor-α
TNFR	Tumor necrosis factor receptor
TTR	Transthyretin
UTR	Untranslated regions
Vip	Vasointestine peptide
Wld^s	Slow wallerian degeneration
WT	Wild type
ZBP-1	Zipcode-binding protein-1

Introduction

1. Historic perspective on axonal regeneration

For many years, the nervous system was seen as unalterable following maturation. Axonal elongation of central nervous system (CNS) axons was thought to be exclusive of the development stage. Ramon y Cajal made the first descriptions on axonal regeneration of peripheral nerves by the end of the XIX century, and went on to characterize the abortive regeneration of CNS axons both in spinal cord and in the cerebral cortex (Ramon y Cajal and May, 1928).

Ramon y Cajal described the peripheral nerve as an environment that could guide and fuel the regrowth of the injured axons. In his own words: "the great influence that the proximity of the peripheral stump has on the growth and orientation of the outgrowing newly formed fibers. We believe it likely that this action is exercised through ferments or stimulating substances formed by the rejuvenate Schwann cells of the distal stump poured out by the regions near the scar. These substances have not only an orienting function, but they are also trophic in character, since the sprouts that have arrived at the peripheral stump are robust, show a great capacity for ramification and grow straight to their goal without vacillations, as though they were following an irresistible attraction" (Ramon y Cajal and May, 1928) (Fig. 1, left image). This description points out the importance of Schwann cells in the regenerative process, and indeed, the ability of Schwann cells to dedifferentiate and support regeneration following injury is one of the most important features of peripheral nervous system (PNS) regeneration (Vargas and Barres, 2007; Gaudet et al., 2011). He then described the difficulty of CNS axons to regenerate, where only in young animals a small and limited ability to regenerate is found: "For our part, by dint only of persistent explorations were we able, finally, to discover unquestionably active production of new fibers, although ephemeral and, therefore, frustrated. Such vicarious sprouting is exclusively observed in young animals (cat and dog of ten to twenty days) and at the levels of the varicosities along the trajectories at the terminal clubs of the axons interrupted inside the white matter (central stumps). Two main varieties are presented:

a. From a thick, terminal (retraction ball) or en passant varicosity arise several fine and pale radiations that get lost in the neighboring territories where they ramify and end in a pale tip. Because it evokes the shape of the tortoise, I named such a singular disposition the testudinoid apparatus.

b. At the frontiers of a necrosed axon segment, the surviving neurofibrils of the neighboring varicosity enter into active proliferation, generating certain tufts of small branches that invade the dead protoplasm, where they end my means of boutons or rings. Because of its shape which somewhat recalls that of the cuttlefish, I baptized such an unusual disposition with the name of cephalopodic apparatus" (Ramon y Cajal and May, 1928) (Fig. 1 right image).

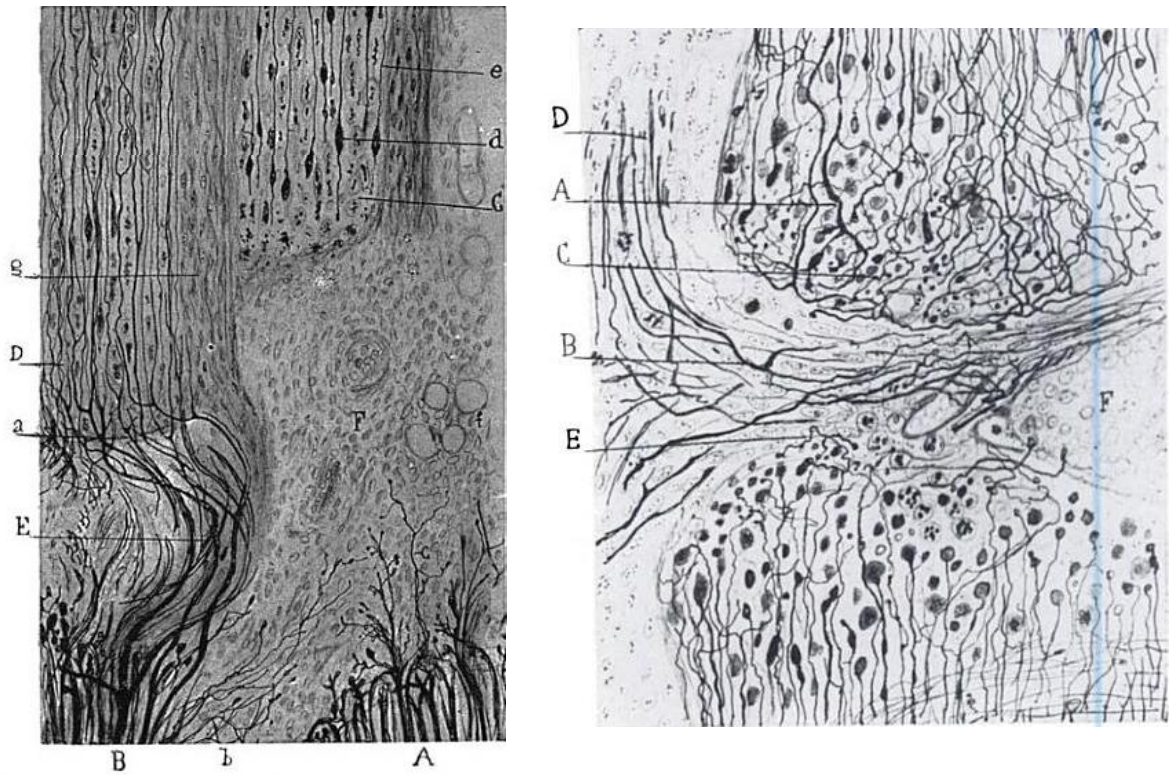

Figure 1. Ramon y Cajal drawings of peripheral and central nervous system injuries. Left: injured sciatic nerve. In the left nerve, both ends are kept together, allowing axonal regrowth to the distal portion, while in the right nerve, the gap created does not allow axonal regrowth. Right: injured spinal cord displaying scar tissue and absence of axonal growth through the formation of retraction bulbs. Adapted from (Ramon y Cajal and May, 1928).

Although many advances have been made in the axonal regeneration field, and despite that several molecular mechanisms underlying axonal growth have been dissected, the general view has not changed greatly since the first descriptions by Ramon y Cajal. To date, despite of the several ongoing clinical trials, still only very limited axonal regeneration is achieved in the CNS. As such, new studies to understand and improve regeneration of CNS axons are of the utmost importance.

2. Axonal elongation during development

During development, axonal elongation occurs both in PNS and CNS. During this process, neurons express a genetic program that allows a robust elongation and the correct interpretation of the guidance cues to reach their post-synaptic targets (Polleux and Snider, 2010). In many ways, regeneration can be viewed, at least in part, as a recapitulation of the developmental process, since axons need to regrow towards their targets. Axonal elongation declines during development due to loss of the neuronal intrinsic ability to elongate (Cai et al., 2001; Goldberg et al., 2002). As such, the developing nervous system has been used as a model to identify genes that may be crucial for the control of axon elongation (Moore et al., 2009).

Throughout development, neural progenitor cells divide asymmetrically, giving rise to a new progenitor daughter cell and a neuron. The newborn unpolarized neurons start migrating to their final destination. During this process, neurons become polarized forming an axon and a leading process that later will form the dendrites (Barnes and Polleux, 2009) (Fig. 2).

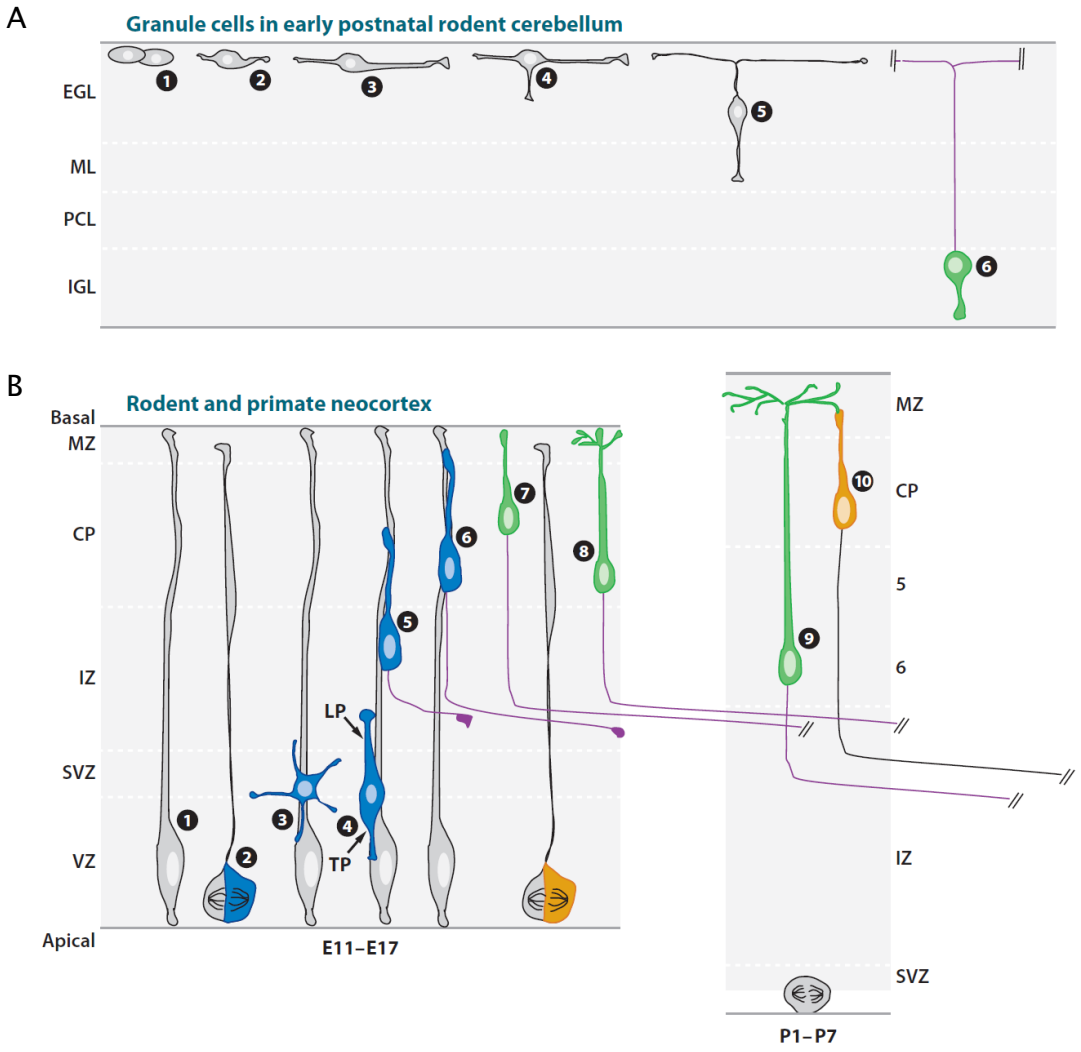

Figure 2. Pattern of the *in vivo* neuronal polarization and migration of cerebellum granule neurons (CGN) and pyramidal cortical neurons. A. Representation of the polarization of the CGN in the mammalian cerebellum. The progenitor cells divide rapidly in the external plexiform layer (EGL, 1) and upon cell cycle exit, start to adopt a bipolar morphology (2). Then, progenitors migrate tangentially with a leading and a trailing process (3). Another process emerges orthogonally from the cell body (4), and becomes the leading process, directing migration towards the inner granule layer (IGL, 5). The trailing processes form a characteristic T-shaped axon (purple), whereas the leading process gives rise to the dendritic domain (green, 6). B. Representation of the radially migrating pyramidal neurons in the mammalian neocortex. Neurons are generated between E11 and E17 in the ventricular zone by asymmetric division of radial glial progenitors. The progenitor cells have a long basal process attached to the basal membrane and a short apical process on the ventricle side (1). Upon division (2), the post mitotic neuron (blue) goes through a multipolar transition where multiple neurites emerge from the cell body (3), before one major process forms in the radial direction and becomes the leading process (4). The neuron initiates a radial migration along the radial glial process and leaves behind a trailing process that elongates tangentially in the intermediate zone (purple, 5). The cell body continues to migrate towards its final destination (the top of the cortical plate), while the axon rapidly elongates (6). The leading process gives rise to the apical dendrite (green, 7), which initiates local branching in the marginal zone (until radial

migration ends). During the first postnatal week, the cell body will then translocate ventrally (8-9) and neurons born at later stages (orange) will bypass them (inside-out accumulation pattern, 10). Adapted from (Barnes and Polleux, 2009).

In this stage, neurons possess a high intrinsic ability to elongate. This ability is transcription dependent and relies on the activity of several signaling pathways, like phosphoinositide 3-kinase (PI3K), phosphatase and tensin homologue (PTEN) and glycogen synthase kinase 3 beta (GSK3β) (Barnes and Polleux, 2009; Polleux and Snider, 2010). Local protein synthesis and degradation also play an important role for correct axonal elongation (Campbell and Holt, 2001). In this context, the ubiquitin-proteasome system emerged as a key player in the control of axon growth during development.

The ubiquitin ligase anaphase promoting complex (APC) and its activator protein, Cdh1 were shown to be important regulators of axon growth during development in post-mitotic neurons (Konishi et al., 2004), acting on the nucleus, limiting axon growth (Stegmuller et al., 2006). A reduction in Cdh1 in CGN leads to an increase in axon length, both *in vitro* and *in vivo* (Konishi et al., 2004) which is achieved by targeting both SnoN and Id2 for degradation (Lasorella et al., 2006; Stegmuller et al., 2006). However, it is not clear if upon completion of development of the nervous system, Cdh1-APC plays a similar role namely, if it limits axon overgrowth in the adult nervous system, or even if it inhibits axonal regeneration.

2.1. Cytoskeletal dynamics in neuron polarization and axonal growth

Upon formation, the immature neurons polarize, giving rise to one axon and several dendrites. Upon polarization, maturation proceeds with axon and dendrite elongation (Polleux and Snider, 2010). Most players responsible for axon initiation and elongation were identified using *in vitro* cultures of hippocampal neurons, a system where the timeline for polarization and elongation are very well defined (Dotti et al., 1988) (Fig. 3).

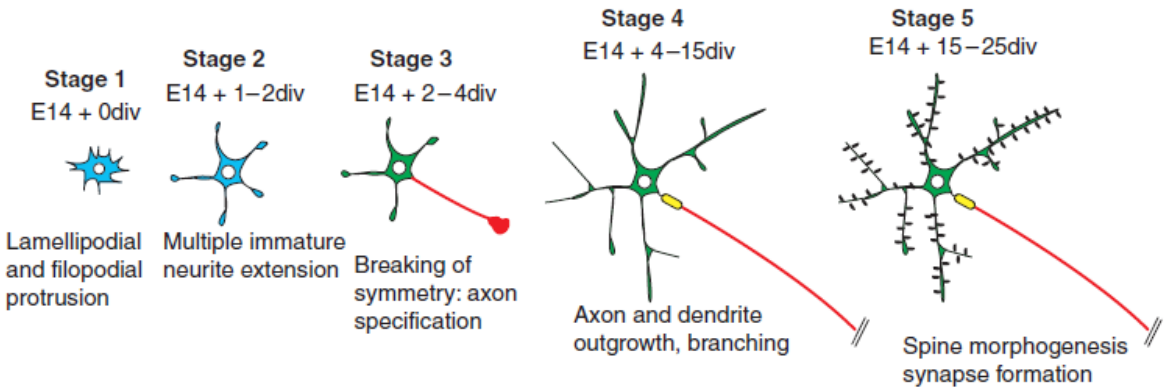

Figure 3. Neuronal polarization of hippocampal neurons *in vitro*. In dissociated cultures of postmitotic hippocampal neurons, stage 1 neurons present an intense lamellipodia and filopodia that leads to the emergence of multiple immature neurites, stage 2. Then a critical step occurs, and neurons become asymmetric and a single neurite grows rapidly originating the axon (red), stage 3. Subsequently there is a rapid elongation of both axon and dendrites (stage 4), and finally neurons present dendritic spines and the axonal initial segment. Adapted from (Polleux and Snider, 2010)

The *in vitro* studies led to the notion that this polarization was mainly due to intrinsic neuronal factors. Cytoskeleton dynamics was found to be crucial for polarization and growth. In immature hippocampal neurons (stage 2), before axon formation, there is local actin destabilization in one of the neurites that will then become the axon. The importance of actin stability was shown by the use of actin-destabilizing agents like lactrunculin B and cytochalasin D that promoted axon formation (Bradke and Dotti, 1999). The local stability of actin is regulated by profilin that promotes actin polymerization and cofilin that promotes actin depolimerization (Witte and Bradke, 2008). Contrary to actin, during axon formation there is microtubule stabilization that is needed for microtubule protrusion. In fact, local microtubule stabilization by the application of taxol is able to induce axon formation (Witte et al., 2008). GSK3β activity is essential for the regulation of microtubule stability. It does so by regulating the activity of several microtubule binding proteins like microtubule-associated protein 1B (MAP1b), collapsin response mediator protein 2 (CRMP-2) and adenomatous polyposis coli protein.

Besides axonal polarization, cytoskeleton dynamics is also important for axonal growth. In fact, microtubule destabilization underlies the formation of a retraction bulb, a structure that impedes axonal growth. Furthermore, stabilizing microtubules with the use of taxol promotes axonal growth (Erturk et al., 2007).

The role of actin dynamics during axonal growth still needs to be further addressed.

2.2. Growth cone, leading the way

The distal tip of the growing axon, the growth cone, is the structure responsible for axonal elongation (Fig. 4). At the growth cone there is the integration of the extracellular cues and this structure is responsible for the elongation through the correct pathway (Jung et al., 2011).

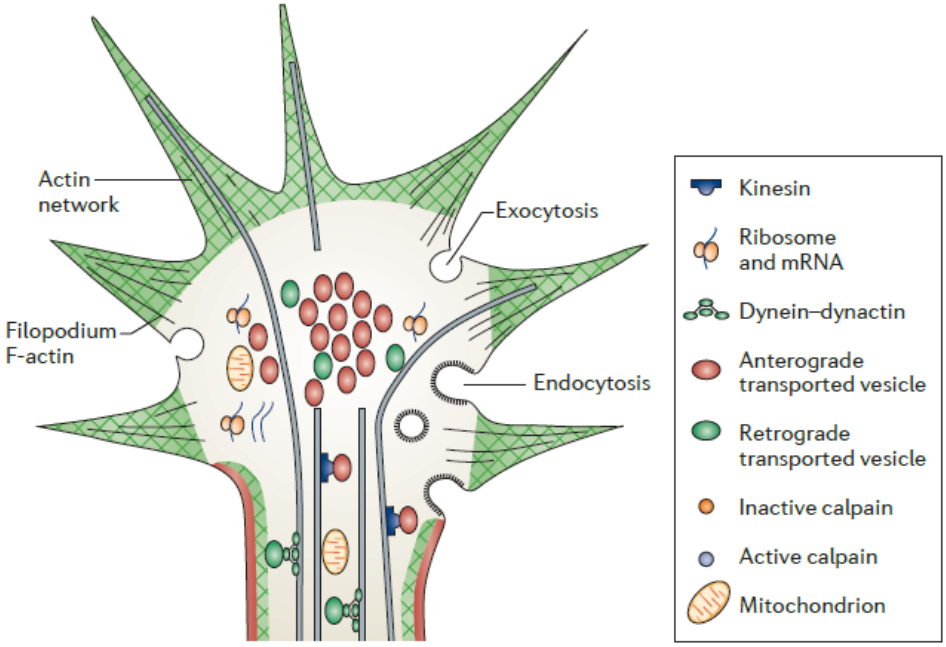

Figure 4. Growth cone structure. The scheme represents a mature growth cone composed of dynamic actin filaments at the periphery, forming the lamellipodia and stable microtubules in the axon shaft. Adapted from (Bradke et al., 2012).

Extracellular cues guide growing axons to reach their correct targets (Fig. 5). Neurotrophins stimulate axonal growth and are required for the transcription of genes important for elongation to take place. Although the initial phase of axonal growth is independent of neurotrophins, the later stages of axonal growth require neurotrophins, and their absence leads to cell death and innervation failure (Polleux and Snider, 2010). Besides neurotrophins, during development the growth cone is exposed to several guidance cues, like semaphorins, ephrins

and netrin (Song and Poo, 1999). The optic nerve formation and development has been the typical model to study the importance and the mechanism of action of these molecules. It was shown that guidance cues influence developing axons by promoting either local protein synthesis (attraction) or degradation (repulsion) (Jung et al., 2011).

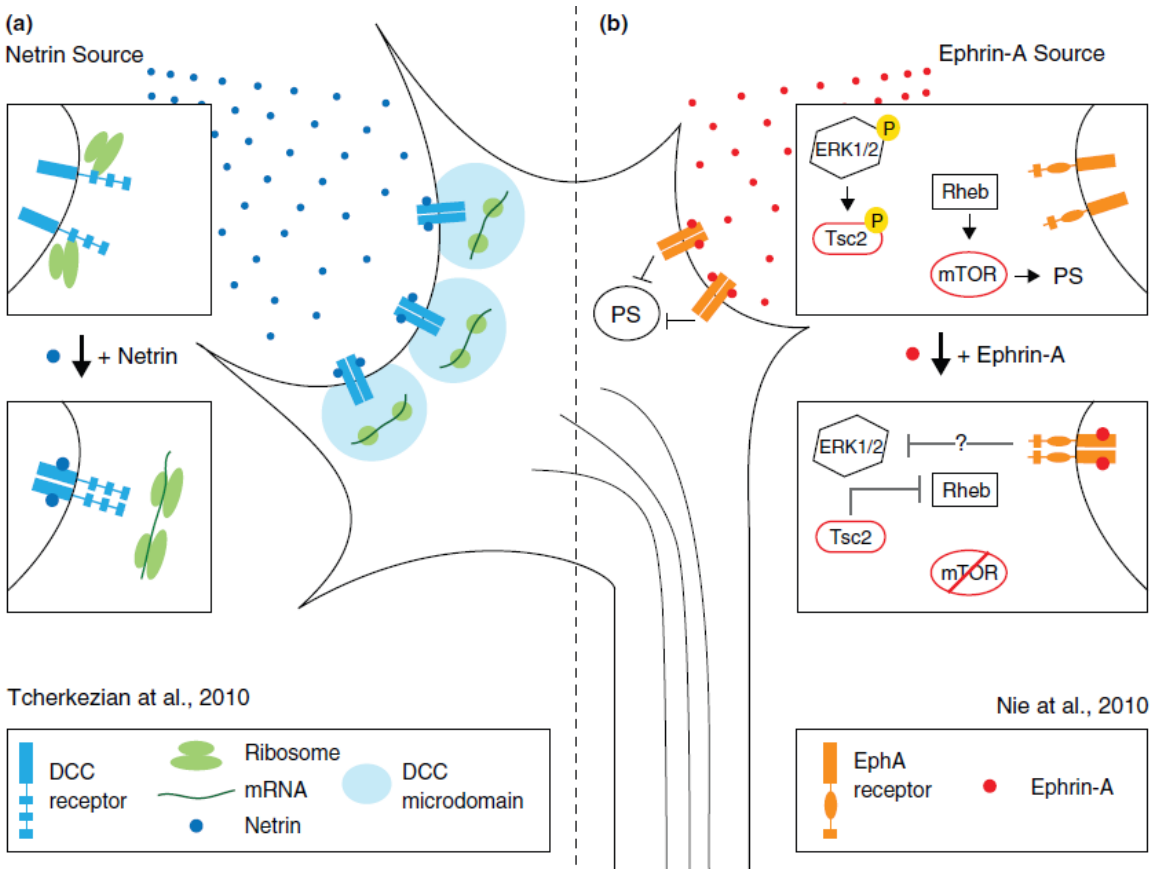

Figure 5. Action of guidance cues in the growth cone. Attractive guidance cues induce growth cone attraction by the stimulation of local protein synthesis (left) and repulsive guidance cues induce repulsion by stimulation of protein degradation (right). Adapted from (Jung et al., 2011).

Furthermore, it has been described that the attractive/repulsive behavior of the extracellular cues is dependent on the cyclic adenosine monophosphate (cAMP): cyclic guanosine monophosphate (cGMP) ratio (Nishiyama et al., 2003). Guidance cues can be divided in cAMP- or cGMP- responsive. In the cAMP-responsive group are included netrin-1, myelin associated glycoprotein (MAG), nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), which attract axons with high levels of cAMP, and repulse axons with low levels of cAMP (Song and Poo, 1999). In the cGMP-responsive group are included semaphorins

and neurotrophin-3 (NT-3), which attract axons with high levels of cGMP, but repulse axons with low levels of cGMP (Song and Poo, 1999; van Horck et al., 2004). Since the levels of cAMP vary from development to adulthood, this mechanism allows understanding how some molecules that attract axons during development become inhibitory in the adult nervous system.

3. Regeneration of PNS axons – axonal regeneration is possible.

Injured PNS neurons are able to regrow to a significant extent and are often used as a model to understand how axonal regeneration can be achieved in the nervous system. This regenerative ability is supported by numerous factors that can be divided in two categories: extrinsic and intrinsic. The extrinsic factors include the role played by the supporting glia and the immune response triggered by the injury. The intrinsic factors comprise the ability of the neurons to express genes that allow their survival and increase their ability to regrow their axons following injury. These are known as regeneration-associated genes (RAGs). Below we will discuss in detail the importance of these mechanisms in the successful PNS regeneration.

3.1. Extrinsic factors – the importance of Wallerian degeneration

Wallerian degeneration was first described in 1850 by Augustus Volney Waller (Waller, 1850) and comprises all the mechanisms that happen in the distal part of the nerves allowing the clearance of the debris and the formation of a favorable environment for axonal regeneration to take place (Fig. 6). Although one may think that once an axon is cut from the cell body it dies immediately actually, following PNS injury, the distal stumps of severed axons are still able to transmit action potentials when stimulated (Luttges et al., 1976) and do survive for a few days before they start degenerating (Gaudet et al., 2011). In young rats the delay between injury and the onset of degeneration is 1 day (Lubinska, 1977) while in humans it takes several days for degeneration to occur (Chaudhry and Cornblath, 1992). The clearance of the distal end of injured nerves is essential to achieve axonal regeneration. Below the mechanisms by which myelin and axonal debris are cleared will be discussed.

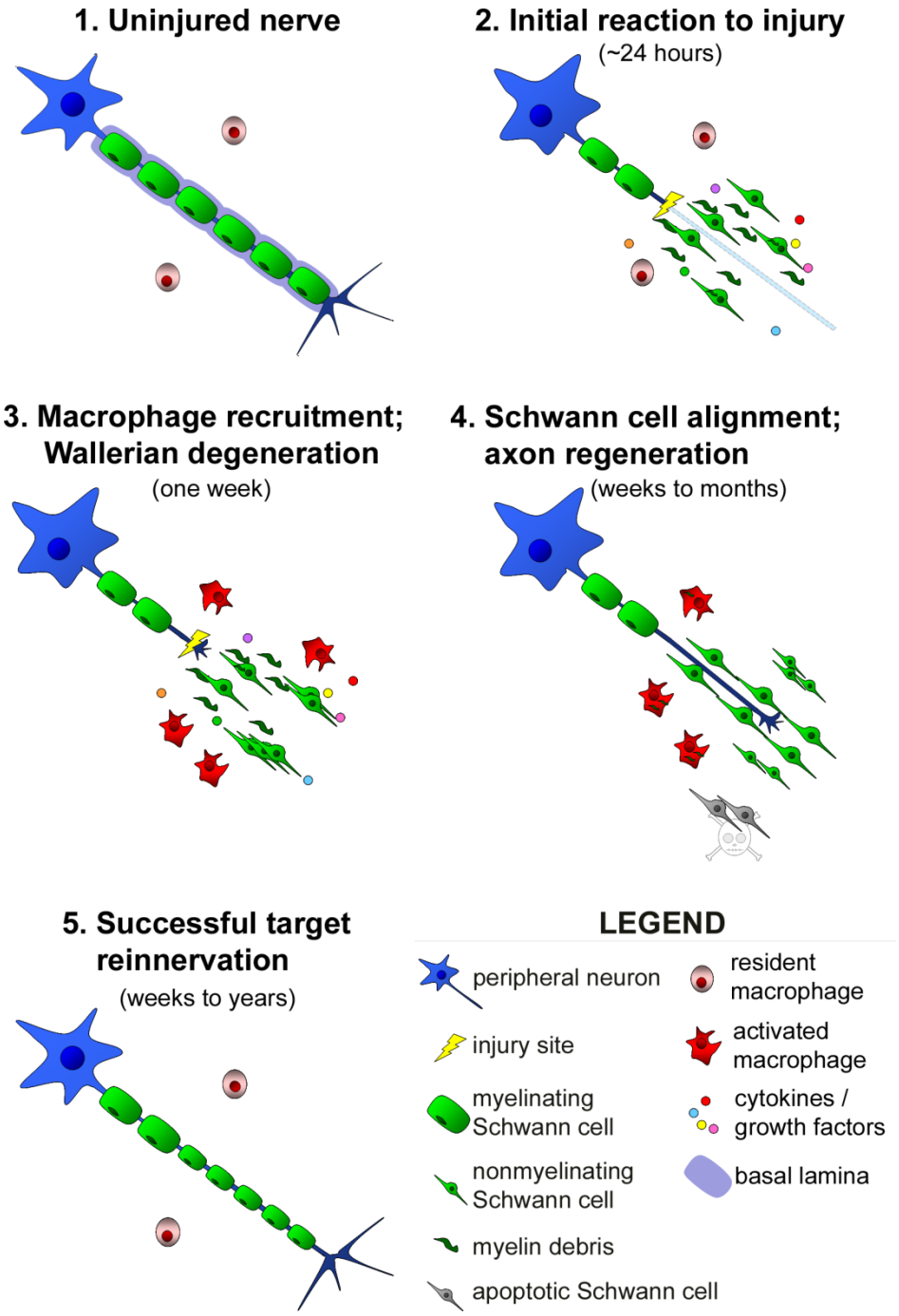

Figure 6. Wallerian degeneration. Following PNS injury there is dying back of the distal axons and Schwann cell dedifferentiation (2). Then macrophages invade the nerves and together with Schwann cells they clear the myelin debris (3). The Schwann cells align forming the bands of Bungner which serve as a rail for the regenerating axons (4) culminating in successful regeneration (5). Adapted from (Gaudet et al., 2011).

3.1.1. Axonal degeneration and break down.

Calcium influx is one of the first signals that an injury has occurred. It leads to calpain activation which triggers the resealing of the membrane of the injured axons (Krause et al., 1994; Howard et al., 1999). Upon injury, membrane disruption promotes a transient calcium influx that activates the intracellular signaling responsible for the membrane reseal (Krause et al., 1994) and for local protein synthesis (Chierzi et al., 2005). Calcium influx activates calcium-dependent enzymes including adenylate cyclase, promoting increased cAMP levels that signal to the downstream effector dual leucine zipper kinase (DLK) promoting cytoskeleton rearrangements needed for growth cone assembly (Ghosh-Roy et al., 2010). Besides cytoskeleton rearrangements, local protein synthesis and degradation are important for growth cone formation. These processes are tightly regulated by the mammalian target of rapamycin (mTOR), p38MAPK and caspase-3 (Verma et al., 2005). Following axonal injury, the correct formation of a growth cone is a key step leading to axonal regeneration.

An elegant study using *in vivo* imaging showed that approximately 20 min following injury, both proximal and distal ends suffer 200-300μm fast degeneration in a process called acute axonal degeneration. Following this process, the proximal end is stabilized and can start regenerating as early as 30h after injury, while the distal ends can persist up to 48h before starting to degenerate (Kerschensteiner et al., 2005).

At the distal part of injured axons, the first visible sign of injury is the axonal membrane beading and swelling. The mechanisms by which the beading and swelling occur are independent of calcium and of the ubiquitin-proteasome system, since they are not inhibited by calcium chelators or by ubiquitin-proteasome inhibitors (George et al., 1995; Zhai et al., 2003). These morphological changes occur before the onset of cytoskeleton disintegration, a process called granular disintegration of the axonal cytoskeleton where the microtubules and neurofilaments are dismantled leading to axonal fragmentation (Gaudet et al., 2011). This process is calcium and ubiquitin-proteasome system-dependent, and it can be inhibited by blocking the ubiquitin-proteasome system (Zhai et al., 2003) or by blocking the ion-sensitive protease calpain (George et al., 1995). These two mechanisms have different roles. The ubiquitin-proteasome system disassembles microtubules, while the calcium-activated calpain response secures neurofilament degradation. Once this process starts, the complete

destruction of the cytoskeleton components into fine debris is completed in one hour (George et al., 1995; Beirowski et al., 2005; Kerschensteiner et al., 2005).

The degenerative process was initially envisaged as a passive process, where axons died given the lack of connectivity to the cell body. It is now known that it is an active process, intrinsic to the axons that can be controlled and delayed. This paradigm shift has emerged with the studies in the slow wallerian degeneration (Wld^s) mouse (Perry et al., 1990), in which the injured distal nerve ends degenerate much slower and are able to conduct action potentials for 2-3 weeks while wild type animals only conduct action potentials for 3 days (Lunn et al., 1989; Tsao et al., 1999). The study of the Wld^s mouse also revealed the importance of axonal degeneration in the regenerative process. The delayed cytoskeleton disintegration is accompanied by a delay in myelin sheath breakdown and macrophage influx, ultimately leading to impaired axonal regeneration (Bisby and Chen, 1990).

3.1.2. The importance of Schwann cells

Schwann cells are the glial cells of the PNS. They play an essential role in nerve physiology since they are responsible for the trophic support of developing and mature neurons, and also for myelin insulation (Bhatheja and Field, 2006). There are two types of Schwann cells: myelinating and non-myelinating Schwann cells. Myelination occurs only in large caliber axons (>1µm), with each Schwann cell myelinating a single axon (Fig. 7, left image). Myelin insulation allows the axon to conduct action potentials much faster. The small caliber axons are ensheathed in a structure called Remak bundle, where a single Schwann cell wraps multiple axons, separating them by a thin layer of cytoplasm (Fig. 7, right image).

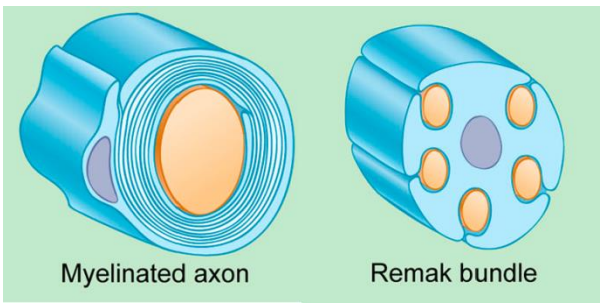

Figure 7. Schwann cell organization in PNS axons. In PNS axons, Schwann cells can either myelinate a single axon (diameter >1µm) or wrap several axons (diameter <1µm) with a thin layer of cytoplasm forming a remak bundle. Adapted from (Salzer, 2008).

The fast and vast response of Schwann cells to PNS injury is one of the main reasons for the successful regeneration of these nerves. Immediately following injury, in the distal part of the severed axons, Schwann cells start to dedifferentiate even before axonal degeneration starts. The mechanism by which Schwann cells sense the axonal injury is not yet known. This dedifferentiation which is also sometimes described as Schwann cell activation following injury, comprises the downregulation of genes related to myelination such as of the genes involved in cholesterol synthesis and structural proteins: P_0, myelin basic protein (MBP) and MAG (Jessen and Mirsky, 2008). At the same time there is the activation of a set of molecules typical from immature Schwann cells, like L1, Neural Cell Adhesion Molecule (NCAM) and glial fibrillary acidic protein (GFAP) (Jessen and Mirsky, 2008), the expression of numerous trophic factors essential for neuronal survival, such as BDNF (Meyer et al., 1992), glial-derived neurotrophic factor (GDNF) (Naveilhan et al., 1997), NGF (Heumann et al., 1987) and the production of several cytokines like leukemia inhibitory factor (LIF) (Curtis et al., 1994), tumor necrosis factor-α (TNF-α), interleukin-1α (IL-1α) (Shamash et al., 2002) and interleukin-6 (IL-6) (Bolin et al., 1995). c-Jun was identified as being essential for the dedifferentiation of Schwann cells. It is activated following injury and it inhibits myelin gene expression (Parkinson et al., 2008). Besides downregulation of myelin genes, c-Jun activation is also needed for GDNF and artemin expression (Fontana et al., 2012). The important role of c-Jun in the Schwann cell response to injury is shown by impairment in axonal regeneration when Schwann cells are depleted of c-Jun (Fontana et al., 2012).

The transcriptional changes suffered by Schwann cells following injury, lead to myelin breakdown and ultimately stimulate proliferation of both myelinating and non-myelinating Schwann cells. Three days following injury Schwann cells start to proliferate and align along the basal lamina forming the bands of Bunger, which provide support and growth factors for the regenerating axons. The aligned Schwann cells produce trophic factors and laminin, an adhesion molecule that is part of the extracellular matrix of basal lamina tubes and is essential for axon growth (Sanes, 1982; Cornbrooks et al., 1983; Chen and Strickland, 2003).

Myelin contains numerous proteins that are inhibitory to axonal growth. The myelin inhibitors that are better characterized are: Nogo (Chen et al., 2000; GrandPre et al., 2000), MAG (McKerracher et al., 1994; Mukhopadhyay et al.,

1994) and oligodendrocyte myelin glycoprotein (OMgp) (Kottis et al., 2002; Wang et al., 2002b). From these three proteins, only MAG is present in PNS myelin (McKerracher et al., 1994). Following injury, there is an accumulation of myelin and of its axonal growth inhibitors that, if not removed, impairs axonal regeneration. This has been shown in animals with slow Wallerian degeneration where the delay in myelin clearance delays PNS regeneration (Brown et al., 1991). Schwann cells and macrophages are responsible for myelin clearance after PNS injury (Vargas and Barres, 2007). Schwann cells degrade myelin using hydrolytic enzymes in intracellular vacuoles (Holtzman and Novikoff, 1965). During the first days following injury, the contribution of macrophages for myelin clearance is small and Schwann cells are responsible for almost all myelin removal (Perry et al., 1995). Schwann cells are able to clear their own myelin, phagocyte extracellular debris and also may function as antigen presenting cells by presenting myelin through major histocompatibility complex II (MHC-II) although this last function is not clear (Holtzman and Novikoff, 1965; Hirata et al., 1999).

3.1.3. The immune response

The immune response in injured PNS nerves is an important feature for their successful regeneration. The immune response is triggered by the expression of several cytokines by Schwann cells (Shamash et al., 2002). Cytokine expression stimulates Schwann cells to express the monocyte chemoattractant protein-1 (MCP-1), an essential component for macrophage recruitment (Subang and Richardson, 2001; Tofaris et al., 2002). In this respect, mice lacking MCP-1 recruit to injured nerves only half of the number of macrophages during Wallerian degeneration (Toews et al., 1998).

Macrophages are also able to clear myelin debris and their invasion into injured nerves completes the myelin removal started by Schwann cells. Actually, myelin removal by macrophages is the last phase of myelin debris clearance. Within 48h after injury, the blood nerve barrier is broken allowing for the invasion of many serum components such as complement and antibodies (Bouldin et al., 1991). These proteins along with MCP-1 expression by Schwann cells lead to the recruitment of macrophages which starts 3 days post-injury and macrophage infiltration is maximum at 14-21 days following injury (Avellino et al., 1995). Myelin degradation by macrophages is mediated by opsonins and is dependent

on the complement system (Bruck and Friede, 1990), such that the invasion of complement components and antibodies into injured nerves is essential, since they "label" myelin debris making them more visible for macrophage removal (Bruck and Friede, 1990).

By far, macrophages are the immune cell type that plays the most important role in PNS regeneration. Other leukocytes play small roles in PNS regeneration. Neutrophils invade injured nerves early after injury and are able to degrade myelin. However their importance in nerve regeneration is not known. In the last phase of the immune response, T cells fine tune immune response producing several pro- and anti-inflammatory cytokines (Gaudet et al., 2011).

3.2 Intrinsic factors

This section served as the basis for the review manuscript: **Mar FM**, Bonni A and Sousa MM (2014). Cell intrinsic control of axon regeneration. EMBO Rep. *In press* doi: 10.1002/embr.201337723 that is reprinted at the end of the introduction section. The successful regeneration of PNS axons is not only due to the removal of myelin negative cues during Wallerian degeneration. PNS neurons also respond to injury by increasing their ability to regrow. Neurons are able to sense an axonal injury many centimeters away from the cell body and to change their transcriptional profile and express several RAGs that increase their ability to regenerate. Bellow I will discuss in detail how PNS axons sense an injury and what are the important features of their regenerative response.

3.2.1. Important neuronal features that allow a regenerative response to be mounted

Neurons are highly polarized cells, where in many cases the cell body and the axon terminal can be separated by many centimeters. Most of the protein synthesis occurs in the cell body following which proteins need to be "shipped" to their correct location. As such, axonal transport is of the utmost importance to deliver proteins along the axons, but also to transmit signals from the axon terminal to the cell body. Although limited, protein synthesis can occur along the axon and in the growth cone during elongation. The local protein synthesis is of extreme importance for local early responses to axonal injury and to growth cone

guidance cues. The importance of axonal transport and local protein synthesis will be discussed in detail.

3.2.1.1. Axonal transport

The axoplasm represents 99% of the neuronal cytoplasm, but protein synthesis in the axon is limited. As such, most of the axoplasm constituents are synthesized in the cell body and then transported to their final destination. Also, axon terminals often receive target derived signals that need to be communicated to the cell body. Given the great length of the axons, those activities pose a great challenge for the neuron. Most of the axonal transport is made along the microtubules in an adenosine-5'-triphosphate (ATP)-dependent way by cargo binding to molecular motors (Fig. 8). In axons, microtubules are uniformly arranged with their plus ends facing the axon terminal (plus-end-out orientation). Two main motors are capable of binding microtubules: kinesin, that is responsible for the transport towards the distal axon tip (plus-end), known as anterograde transport and dynein that is responsible for the transport from the axon tip to the cell body (minus-end), known as retrograde transport (Guzik and Goldstein, 2004).

Besides kinesin and dynein, myosin is another motor performing axonal transport. Contrary to kinesin and dynein that move along microtubules, myosin moves along actin filaments (Seabra and Coudrier, 2004). Myosin is incapable of long distance transport, a function performed by kinesin and dynein. Instead, it is responsible for short distance transport, like local insertion of proteins in the plasma membrane. It also interacts with neurofilaments being responsible for their organization (Bridgman, 2004).

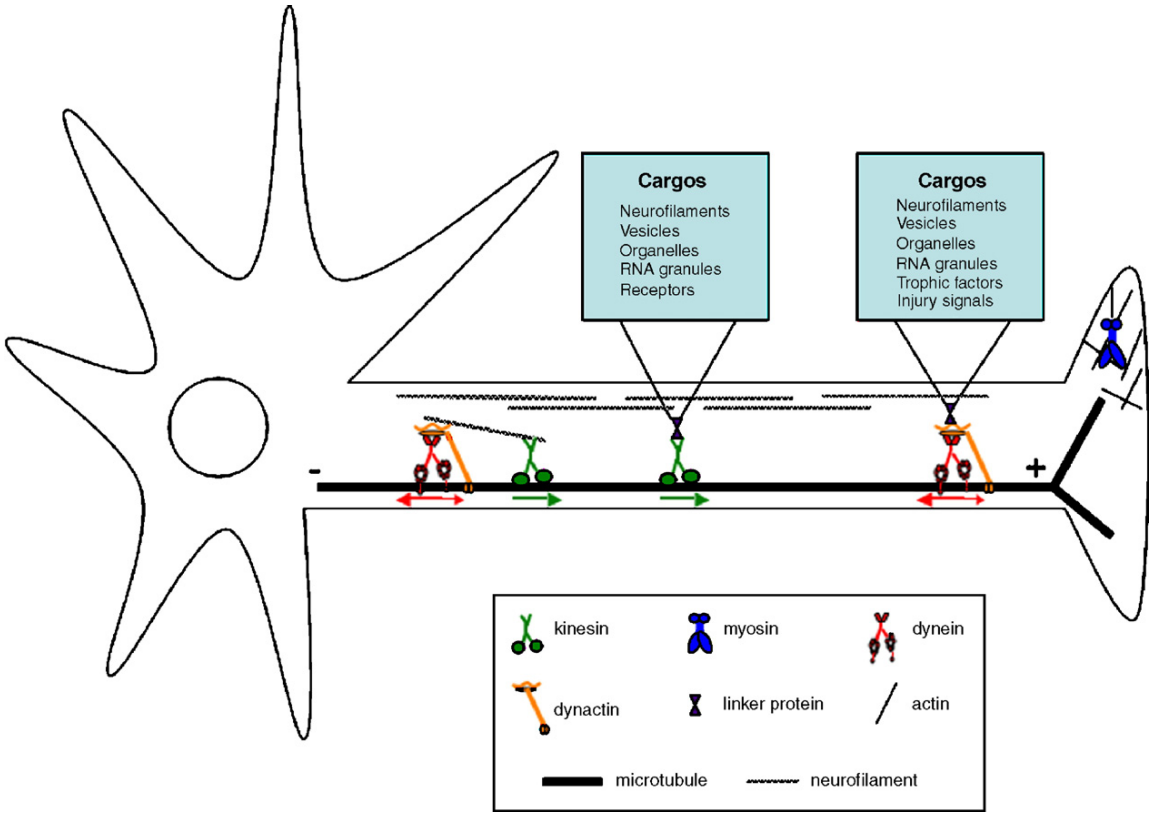

Figure 8. Axonal transport. Kinesin is responsible for the anterograde transport and dynein for the retrograde transport. Both move along microtubules. Myosin moves along actin filaments and is responsible for small range transport. Adapted from (Chevalier-Larsen and Holzbaur, 2006).

3.2.1.1.1. Anterograde transport

The anterograde transport is responsible for axon maintenance by supplying it with structural, synaptic and cytosolic proteins like glycolytic enzymes, vesicles and membranous organelles like mitochondria. Most studies characterizing the different components of anterograde transport were made during the 1980s by radiolabeling newly synthesized proteins in laboratory animals and then by chasing them along the axons (Grafstein and Forman, 1980; Wujek and Lasek, 1983; Lasek et al., 1984). The different transport rates observed suggested the existence of at least two different mechanisms of anterograde transport. The first direct evidences came 20 years later with the development of techniques that allowed the direct observation of cargoes moving in living cells (Kaether et al., 2000; Ligon and Steward, 2000; Wang and Brown, 2001, 2002). In the axon, the anterograde transport of proteins is essentially done by the slow component of axonal transport which is divided in slow component a (SCa), that is responsible for the transport of neurofilament proteins, tubulin and microtubule-associated

proteins at a rate of 0,2-1 mm/day; and the slow component b (SCb) that transports glycolytic enzymes and actin, among others, at a rate of 2-8 mm/day (Lasek et al., 1984; Brown, 2003). Vesicles and membranous organelles are transported in the fast component at 50-400 mm/day (Lasek et al., 1984; Brown, 2003). Surprisingly, the motors and the kinetics of both slow and fast components of axonal transport are similar and the different average rates are explained by an intermittent behavior of cargoes during transport (Brown, 2003). Slow transport components, although traveling at the same rate as the fast component, spend more time in a stationary stage along the way resulting in a lower average rate (Brown, 2003; Roy et al., 2007). These findings raised new questions such as: How is the intermittent behavior controlled? What triggers movement and stoppage? These questions remain unanswered.

Kinesin is a superfamily composed of more than 45 kinesins divided in 15 families (Fig. 9). In axonal transport, kinesin1 is the most relevant and studied kinesin family. This family is coded by 3 different genes: KIF5A, KIF5B and KIF5C (Hirokawa et al., 2009).

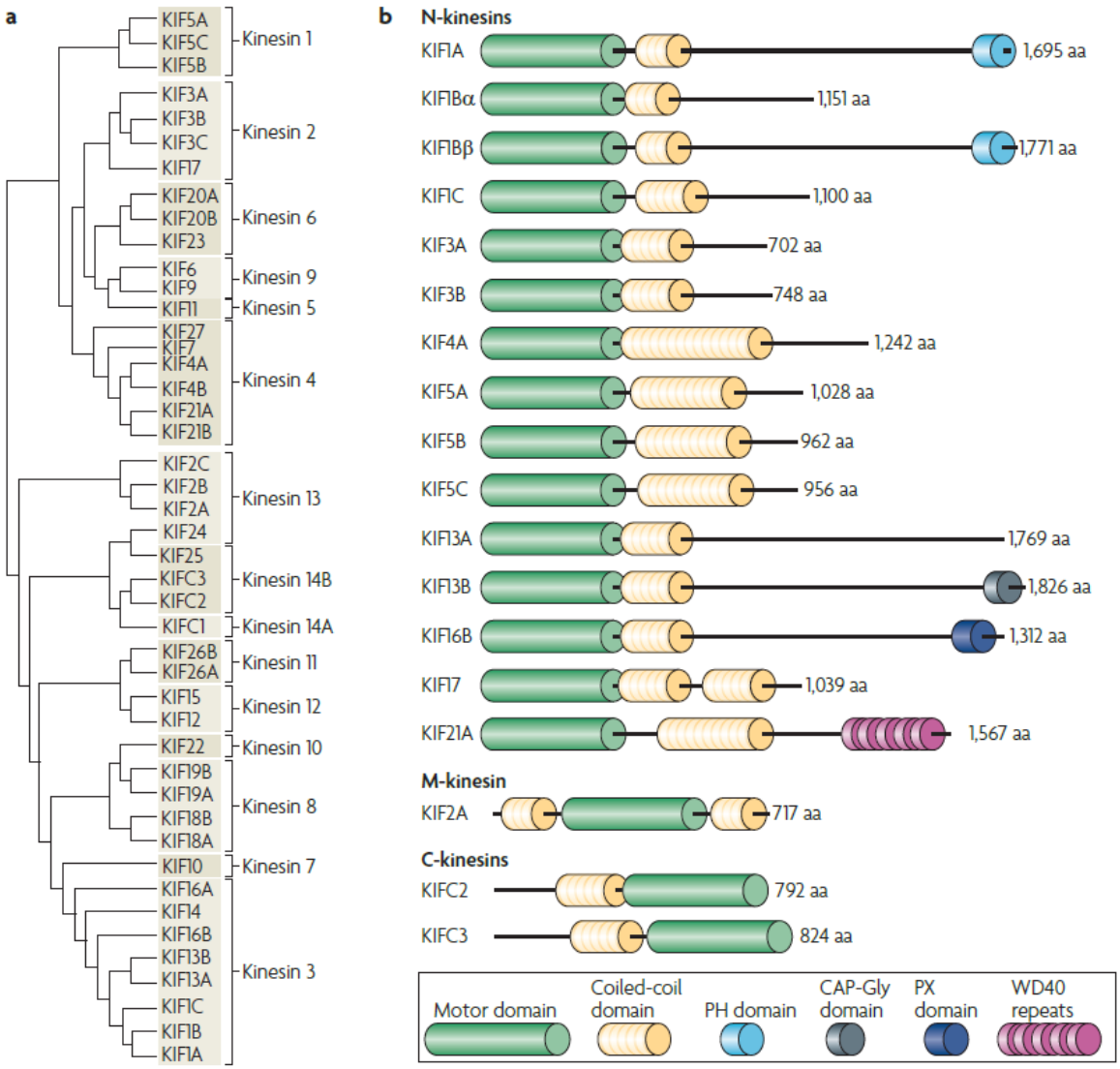

Figure 9. Kinesin protein family. Adapted from (Hirokawa et al., 2009).

KIF5B is ubiquitously expressed, while KIF5A and KIF5C are neuron specific. Kinesin1 generically known as Kinesin heavy chain, usually binds cargos through adaptor proteins – kinesin light chains – forming a tetramer composed of two heavy chains and two light chains (Hirokawa et al., 2009) (Fig. 10). ATP hydrolysis provides the required energy to generate the motile force (Hirokawa et al., 2009).

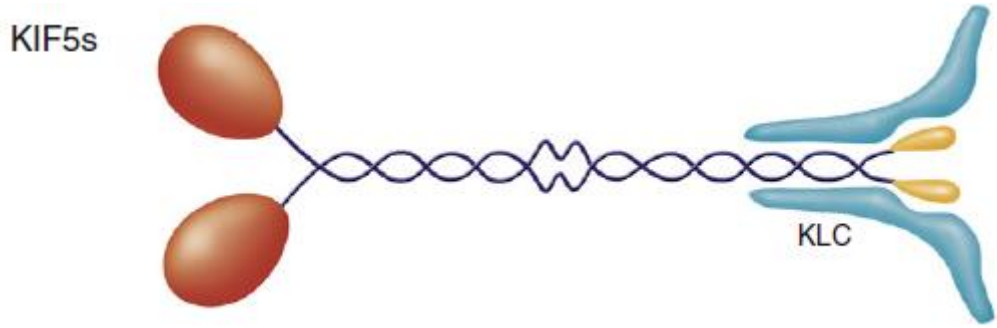

Figure 10. Kinesin structure. Kinesin is composed by two heavy chains and two light chains. Adapted from (Hirokawa et al., 2010).

Besides axonal maintenance, anterograde transport is of particular importance during axonal regeneration since it supplies the necessary proteins for this process, particularly the structural components tubulin, actin and neurofilament (Tashiro and Komiya, 1992; Jacob and McQuarrie, 1996). In fact the speed of axonal regeneration is similar to the rate of the slow component of anterograde transport, confirming the idea that the anterograde transport supplies regrowing axons (Wujek and Lasek, 1983).

3.2.1.1.2. Retrograde transport

Retrograde transport is vital for neuronal survival. It encompasses the transport of Trk receptors activated through signals released by the axonal targets, such as neurotrophins (Heerssen et al., 2004). The retrograde transport of these signals is achieved by the formation of signaling endosomes that are linked to the motor dynein and transported to the cell body where they activate gene expression of survival genes (Delcroix et al., 2003; Ye et al., 2003). Cargos bind dynein through adaptor proteins. Dynactin is the main cargo adaptor and is essential for an efficient dynein-mediated transport (Kardon and Vale, 2009). The dynein/dynactin complex is well described (Fig. 11) for its role in retrograde transport, however it has been reported that under specific circumstances, this complex may have bidirectional movement (Ross et al., 2006).

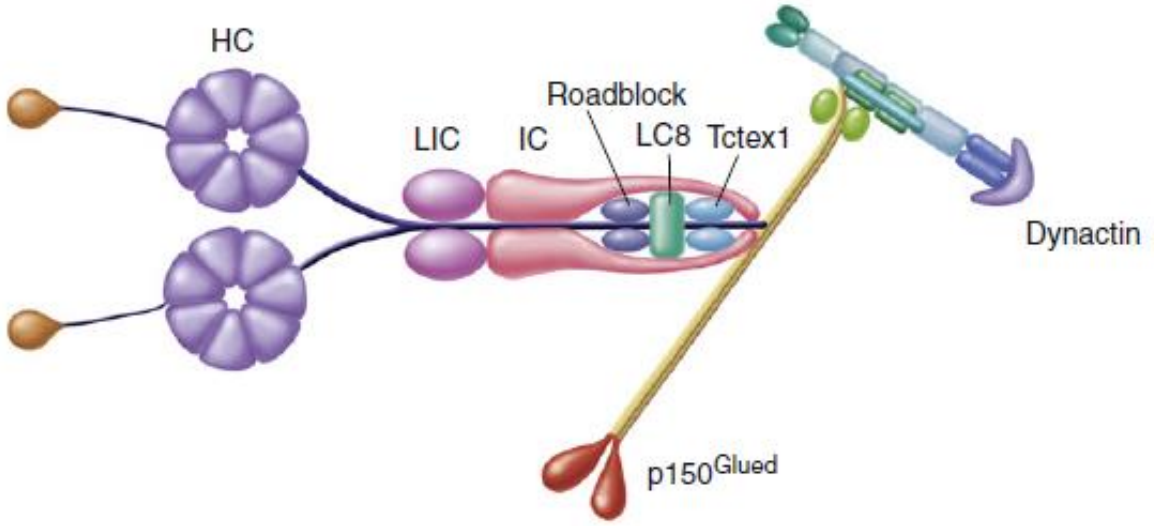

Figure 11. Dynein/dynactin complex structure. Dynein is composed by two heavy chains, two light-intermediate chains, two intermediate chains and two light chains that bind to dynactin for cargo transport. Adapted from (Hirokawa et al., 2010).

The importance of retrograde transport is shown by the fact that its disruption leads to the onset of neurodegeneration (LaMonte et al., 2002; Hafezparast et al., 2003). In fact, in many neurodegenerative diseases such as Amyotrophic lateral sclerosis (ALS), defects in retrograde transport are found even before the first symptoms appear (Kieran et al., 2005; Ligon et al., 2005). Retrograde transport is extremely important to signal injury following nerve lesion. Upon injury, there is the local activation of injury signals that are linked to dynein (Hanz et al., 2003). This linkage ensures the retrograde transport of the injury signals to the cell body (Fig. 12) where they can signal the injury and trigger a regenerative response (Ambron et al., 1992; Schmied and Ambron, 1997; Hanz et al., 2003; Perlson et al., 2005).

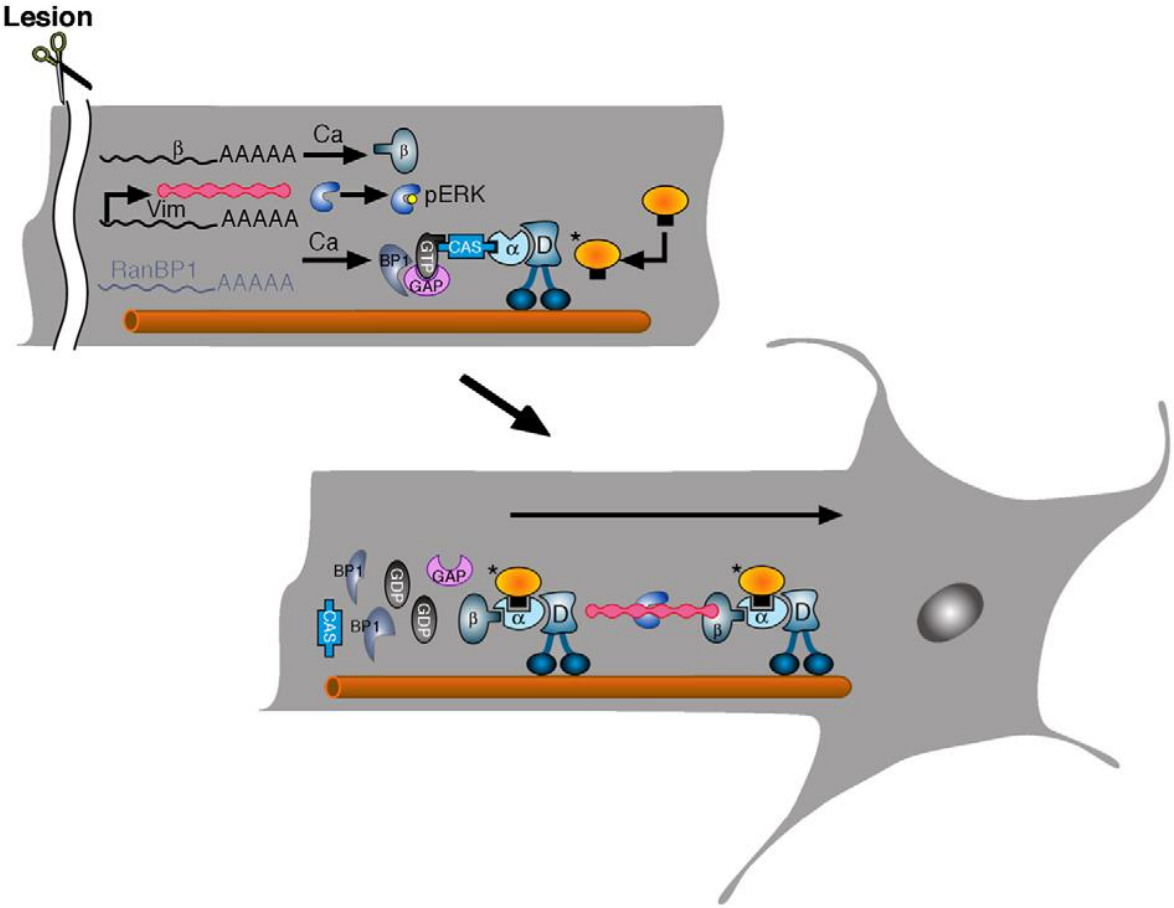

Figure 12. Retrograde transport of injury signals. Following PNS injury there is local activation of injury signals and local translation of proteins that link the activated injury signals to the retrograde transport machinery. Adapted from (Rishal and Fainzilber, 2010).

3.2.1.1.3. Defects in axonal transport as the cause of pathological conditions

Several neurodegenerative diseases present defects in axonal transport, either retrograde or anterograde. These defects can happen either due to mutations in the motors, microtubule destabilization or other undisclosed mechanisms. Charcot-Marie-Tooth type 2A is caused by a single point mutation in KIF1Bβ gene, which impairs the axonal transport of synaptic vesicles, resulting in neurodegeneration (Roy et al., 2005). Mutations in dynactin have been implicated in the disruption of the dynein/dynactin complex, resulting in retrograde transport defects and motor neuron cell death (Chevalier-Larsen and Holzbaur, 2006). Although axonal transport defects have been found in other neurodegenerative diseases such as Alzheimer's, Huntington and ALS (Roy et al., 2005), whether these defects are a cause or a consequence of neurodegeneration is still a matter of debate.

Table 1. Axonal transport defects in human disease. Adapted from (Chevalier-Larsen and Holzbaur, 2006).

	Disease name	Mutant protein	Cellular pathology relevant to transport deficits
Motor mutations	CMT type 2A	KIF1B beta	reduced transport of synaptic vesicle proteins
	spastic paraplegia	KIF5A	altered microtubule/motor interaction
	CFEOM type1	KIF21A	predicted to disrupt anterograde transport of vesicles
	distal SBMA	$p150^{Glued}$	reduced microtubule/$p150^{Glued}$ interaction p150 aggregation
Cytoskeletal mutations	CMT type 2	HSP27	disruption of neurofilament formation p150 mislocalized and aggregated
	HRD	TBCE	disruption of microtubule stability
	CMT type 2	NF-L	disruption of anterograde and retrograde transport
Neurodegenerative diseases involving axonal transport	Huntington's disease	huntingtin	disruption of anterograde and retrograde transport axonal swellings
	SBMA	AR	disruption of anterograde and retrograde transport
	Alzheimer's disease	APP/PS1	disruption of anterograde transport axonal blockage
	ALS	SOD1	deficits in anterograde and retrograde transport dynein/SOD1 aggregates

3.2.1.2. Local protein synthesis in the axon

Protein synthesis was thought to be exclusive of the neuronal cell body. This notion was supported by the lack of evidence of messenger RNA (mRNA) and translation machinery on mature axons (Lasek et al., 1973). However, with the development of more sensitive techniques it was found that indeed axons possess both mRNA and translation machinery (Tennyson, 1970; Giuditta et al., 1980; Giuditta et al., 1986; Giuditta et al., 1991; Bassell et al., 1998). Moreover, metabolic labeling studies showed that axons without cell body are able to synthesize proteins *de novo* (Tobias and Koenig, 1975; Koenig and Adams, 1982; Koenig, 1991; Eng et al., 1999). Initially, a small number of mRNAs were identified in the axon, but the number of axonally localized mRNAs has been growing considerably with the development of more sensitive techniques (Jung et al., 2012).

The targeting of mRNAs to different axonal compartments is a mechanism that allows the regulation of local protein synthesis. Current techniques have contributed greatly to the identification of novel axonal mRNAs. It also allowed the identification of crucial changes of axonal mRNA content from development to adulthood (Vogelaar et al., 2009; Gumy et al., 2011).

Local protein synthesis is particularly important for accurate fast responses when there is not enough time to communicate with the cell body. In fact, during

development, growing axons are exposed to several guidance cues, like netrin-1, ephrin B, semaphorin 3A, NGF and BDNF, which need to be interpreted rapidly for the correct pathfinding (Lin and Holt, 2008; Jung et al., 2012). The growth cone is the structure responsible for the integration of the environment signals in a process dependent on local protein synthesis (Campbell and Holt, 2001; Ming et al., 2002). Although CNS axons are able to synthesize proteins in the growth cone during development, studies with adult CNS axons fail to show the presence of ribosomes, suggesting that adult CNS axons are not able to synthesize locally proteins or that this ability is very limited (Steward and Ribak, 1986; Verma et al., 2005). This can underlie in part their limited ability to regenerate. In contrast, adult PNS axons do possess ribosomes distributed unevenly along the axoplasm, close to the plasma membrane (Koenig et al., 2000; Li et al., 2005; Kun et al., 2007). Schwann cells have also been suggested as a source of ribosomes for PNS axons following injury, indicating that Schwann cells may promote local protein synthesis (Court et al., 2008; Twiss and Fainzilber, 2009). Protein synthesis is generally decreased with axonal ageing, that coincides with reduced axonal regeneration potential (Gumy et al., 2010). This evidence further reinforces the importance of local axonal synthesis in regeneration.

As such, besides its importance for axonal steering during development, local protein synthesis has been shown to be important following peripheral nerve injury by two different mechanisms. It enables the synthesis of vimentin and importin-β that can be linked to the retrograde transport machinery along with locally activated proteins and transported back to the cell body where they can trigger a robust regenerative response (Hanz et al., 2003; Perlson et al., 2005). Also, the initial steps of axonal regeneration are achieved by local protein synthesis, since the arrival to the injury site of proteins synthesized in the cell body can take a few days. As such, the formation of a growth cone, an essential structure for successful regeneration, as well as the provision of the first building blocks of the regrowing axons is obtained by local protein synthesis (Verma et al., 2005; Willis and Twiss, 2006; Gumy et al., 2010).

Axonal mRNAs face great challenges: they need to be actively transported, stored and protected from degradation at their final destination. The RNA-binding proteins play an essential role in this process, by binding to cis-elements in the 5'- or 3'- untranslated regions (UTR). Upon RNA binding they control its transport, stability and translation (Bassell and Kelic, 2004; Patel et al., 2012). The best

studied mRNA with axonal localization is β-actin. Its mRNA is controlled by the zipcode-binding protein-1 (ZBP-1) that binds to a cis element in the 3'-UTR. This cis element in the 3'-UTR is essential for local translation of β-actin in response to guidance cues (Leung et al., 2006; Yao et al., 2006). The importance of ZBP-1 has been shown by the observation that reduced ZBP-1 activity leads to decreased axon regeneration (Donnelly et al., 2011). Additionally, the overexpression of β-actin 3'UTR competed *in vivo* with other ZBP-1 cargo mRNAs such as growth associated protein 43 (GAP-43) (Yoo et al., 2013). Recently, it has also been shown that axonal translation of β-actin supports axon branching, while that of GAP-43 promotes elongation of sensory neurons (Donnelly et al., 2013).

In summary, the recent findings support that although local axonal protein synthesis is limited, it is of great importance to axonal regeneration.

3.2.2. Injury mechanisms

The mechanism by which an axon can "warn" the cell body that it has been injured is a question that puzzled neuroscientists for many years. Elegant studies made in Aplysia neurons in the 90's described the first injury signals capable of increasing the regeneration ability (Ambron et al., 1995; Ambron et al., 1996). Since then, many advances were made in how PNS axons are able to sense an injury, although many of the proposed signals still lack robust experimental evidence (Fig. 13).

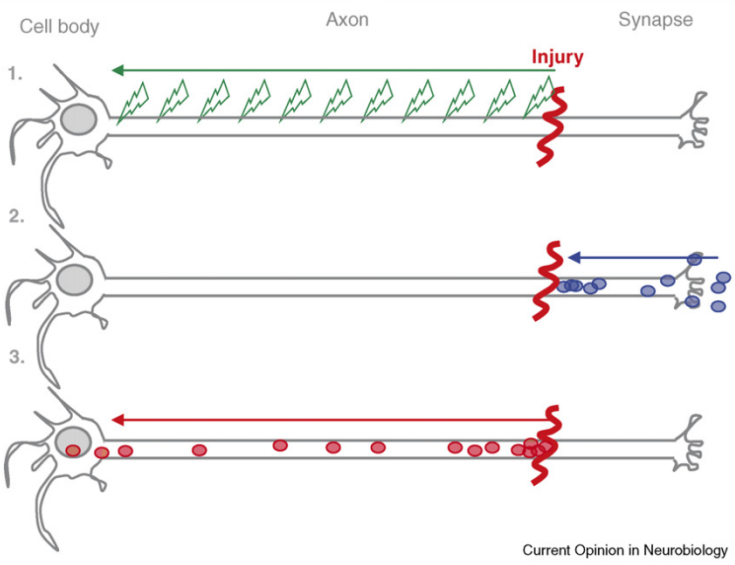

Figure 13. Injury mechanisms following PNS injury. Following PNS injury there is neuronal depolarization (1), interruption of transported target derived signals (2) and local activation and transport of injury signals (3). Adapted from (Abe and Cavalli, 2008).

3.2.2.1. Axonal depolarization

Upon injury, axons are depolarized and this depolarization travels along the axon to the cell body. The depolarization is triggered by an inversion in the Calcium/Sodium flux. Besides depolarization, Calcium influx is essential for the resealing of the axonal membrane (as described above), local transformation of the cytoskeleton to form a growth cone and local activation of protein synthesis (Chierzi et al., 2005; Erez et al., 2007; Kamber et al., 2009; Bradke et al., 2012). In *C. elegans* sensory neurons, the amplitude of the axonal calcium waves correlates with the extent of regeneration, and conversely inhibition of voltage-gated calcium channels, or of calcium release from internal stores, reduces the regenerative growth of axons (Ghosh-Roy et al., 2010). Although the effects on neurons are not consensual, the use of weak electrical stimulation has been shown to improve both the regeneration of motor (Brushart et al., 2002; Al-Majed et al., 2004) and sensory neurons (Udina et al., 2008).

More recently it has been shown that strong electrical stimulation of dorsal root ganglia (DRG) neurons inhibits their axon outgrowth. Loss of electrical activity following PNS lesion due to a decrease in the L-type voltage-gated Ca^{2+} channel was described as an important signal to increase axonal regeneration

(Enes et al., 2010). These results suggested that the electrical activity may be a negative signal that once lost can trigger the injury response in DRG neurons.

In summary, axonal depolarization is thought to be the first signal of injury. However it is seen as a transient signal that, if not followed by other injury signaling mechanisms, cannot trigger a robust, sustained regenerative response.

3.2.2.2. Negative injury signals

During development axons elongate until they find their targets. Once an axon finds its target, the neuron receives several target-derived factors through retrograde transport that repress the elongation machinery of neurons converting the growth cone into a presynaptic terminal.

Upon injury, the neurons are disconnected from their targets and there is an interruption of the normal supply of target-derived signals. This decrease in the supply of target derived signals is thought to alleviate the repression of axon elongation allowing regeneration. These signals are known as negative injury signals since their absence can trigger a regenerative response. It has been shown that neurotrophins are dramatically decreased in DRG neurons following injury (Raivich et al., 1991). Although neurotrophins fulfill many of the requirements for a negative injury signal, their role in nerve regeneration is still unclear. In accordance with a negative injury signal function, it has been described that administration of NGF to injured nerves could delay regeneration (Gold, 1997). However, in other studies, the administration of neurotrophins following injury was linked to an improvement in PNS regeneration (Molteni et al., 2004). Other studies have also described that PNS regeneration may be independent of NGF (Diamond et al., 1992; Tannemaat et al., 2008).

The notion that naïve neurons continuously receive signals that repress axonal growth that are relieved following PNS injury is an appealing idea. The identification of these signals may lead to the development of new methods to improve axon regeneration. However, this hypothesis still lacks robust experimental evidence and so far no clear negative injury signals have been identified.

3.2.2.3. Positive injury signals

The first evidence that axons can produce molecules capable of triggering a regenerative response following injury emerged in the 90s using Aplysia neurons as a model. It was shown that injecting axoplasm extracted from injured nerves in naïve neurons, an injury-like behavior was triggered in the injected neurons. By labeling the injured nerves with γ^{32}P ATP there was evidence that these signals were dependent on phosphorylation (Ambron et al., 1995). Combining these results with previous data on a SV40-type nuclear localization signal (NLS) that targeted axonal proteins to the nucleus through the retrograde transport machinery (Ambron et al., 1992; Schmied and Ambron, 1997), the authors proposed that under normal circumstances, the signal proteins have an hidden NLS, that after injury is exposed by phosphorylation leading to targeting of the signal to the nucleus triggering a response to injury. The importance of retrograde transport of NLS proteins was shown later when an NLS synthetic peptide was injected into the injured nerve precluding the increase in the regeneration capacity by competition with the intrinsic signals (Hanz et al., 2003).

A decade later, the molecular mechanisms underlying the activation of positive injury signals and for their transport were described, but the initial idea was maintained. One of the most important features added to the injury signaling mechanism was the importance of local translation in injured axons. The concept that axons are able to synthesize proteins allowed to understand how a protein containing a NLS is able to undergo retrograde transport only following injury. The NLS-mediated import of proteins to the nucleus is mediated by their binding to importins (Gorlich and Kutay, 1999). It is also important to note that NLS proteins bind with low affinity to importin-α, but with high affinity to importin-α/β heterodimers (Kohler et al., 1999). In normal nerves only importin-α is present making it unlikely that NLS proteins are retrogradely transported to the nucleus. However, following injury, there is the local translation of importin-β. The synthesis of importin-β allows for the formation of importin-α/β heterodimers which bind to NLS and link those proteins to the retrograde transport machinery through dynein binding (Hanz et al., 2003). Recently, it has been shown that the axonal localization of importin-β mRNA is essential for the correct assembly of the retrograde transport machinery of injury signals (Ben-Tov Perry et al., 2012). Besides importin-β, RanBP1 is also locally synthesized following injury and able to

dissociate RanGTP from importins, allowing the binding of new importin-α/β heterodimers to dynein (Yudin et al., 2008).

So far, the study of rodent injured sciatic nerves led to the identification of three injury signals that are locally activated and retrogradely transported to the cell body: the extracellular signal regulated kinase (ERK) (Hanz et al., 2003; Perlson et al., 2005), c-Jun N-terminal kinases (JNK) (Cavalli et al., 2005) and signal transducer and activator of transcription 3 (STAT-3) (Ben-Yaakov et al., 2012). The local activation of ERK and STAT-3 following sciatic nerve injury has been shown (Sheu et al., 2000), but only recently the molecular mechanisms by which these proteins are translocated to the cell body and induce a regenerative response were dissected (Perlson et al., 2005; Ben-Yaakov et al., 2012). Below I will describe the details behind ERK, JNK and STAT-3 function.

ERK is activated by phosphorylation immediately following injury and it is then coupled to the retrograde transport machinery (Hanz et al., 2003). This process is dependent on the local synthesis of vimentin following injury. Vimentin is able to bind both pERK and importin-β linking pERK to the retrograde transport machinery (Fig. 14). pERK is rapidly transported and approximately 20h following injury it reaches the cell body where it leads to activation of E twenty-six like transcription factor 1 (Elk-1). Moreover, impeding the retrograde transport of pERK following injury decreases the subsequent regenerative response (Perlson et al., 2005).

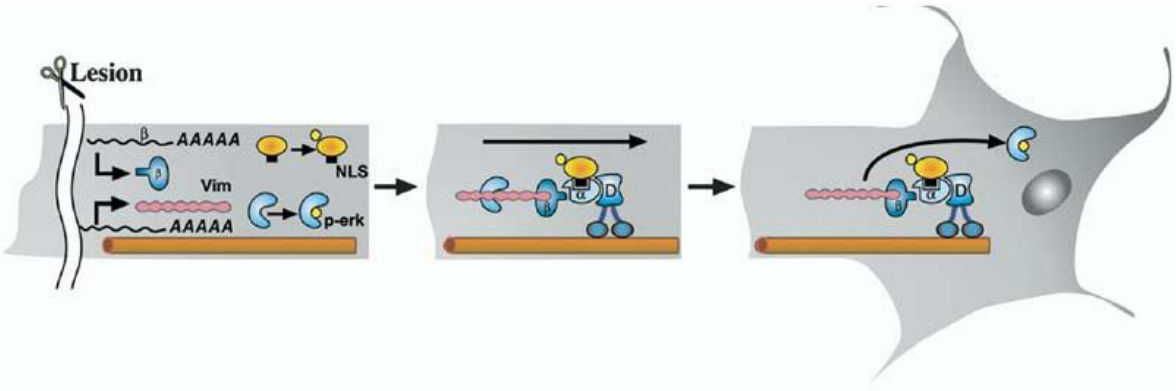

Figure 14. Retrograde transport of pERK following PNS injury. Adapted from (Perlson et al., 2005).

In uninjured nerves, JNK interacts with sunday driver (syd) and both are transported in axonal vesicles, both retrogradely and anterogradely (Cavalli et al., 2005). Following injury JNK is phosphorylated within an hour and there is an

increase in the amount of syd that interacts with dynein. This results in an increase of the retrograde transport of vesicles containing pJNK and Syd leading to an increase in pJNK in the neuron cell body (Cavalli et al., 2005) and to the activation of the transcription factor c-jun, increasing the expression of genes required for regeneration (Davis, 2000; Lindwall et al., 2004).

Another described positive injury signal is STAT-3. STAT-3 is a transcription factor of the JAK-STAT pathway. Following PNS injury there is a local activation of STAT-3 and nuclear translocation in both motor and sensory neurons occurs (Lee et al., 2004). This activation is important to increase the regeneration ability of DRG neurons (Qiu et al., 2005; Miao et al., 2006). More recently it was shown that in DRG neurons, STAT-3 activation is important to initiate the regenerative process. In the absence of STAT-3, injured neurons have a prolonged lag in the initiation of regeneration but can then regenerate at normal rate (Bareyre et al., 2011). STAT-3 locally synthesized and activated is linked to dynein through interaction with importin-α5, allowing STAT-3 to reach the cell body peaking at 18h following injury (Ben-Yaakov et al., 2012) (Fig. 15). Moreover, DLK, a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family that can activate JNK and p38 (Fan et al., 1996), was identified as being important for the retrograde transport of pSTAT-3 following injury. DLK KO neurons present decreased regeneration following PNS injury due to their failure in the retrograde transport of pSTAT-3 (Shin et al., 2012). Surprisingly, Ben-Yaakov et al did not find any impairment in the regeneration of DRG neurons that lack STAT-3 activation. Instead, STAT-3 activation was found to be important in neuronal survival to injury (Ben-Yaakov et al., 2012).

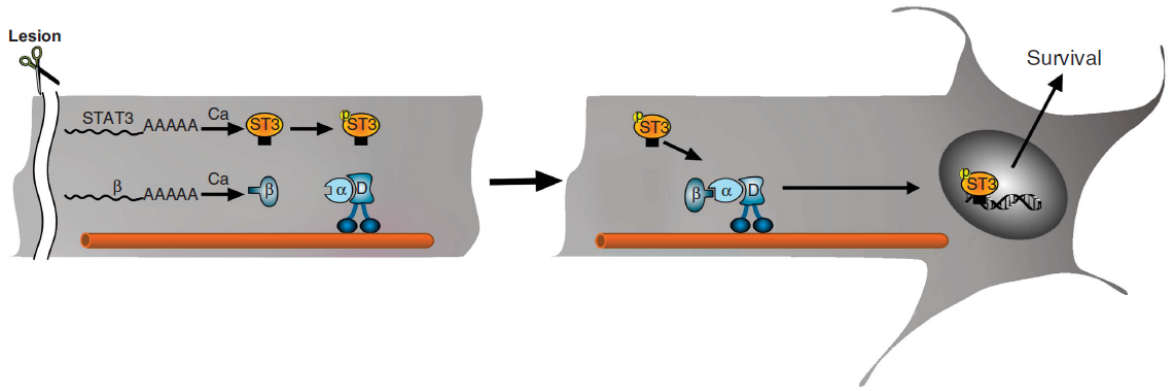

Figure 15. Retrograde transport of pSTAT-3 following PNS injury. Adapted from (Ben-Yaakov et al., 2012).

The transport of activated injury signals for such a long distance as an entire axon represents a great challenge to the cell. Protein phosphorylation is a reversible process. Cells need to protect the phospho active signals to avoid the inactivation of the signals during their course. The function of vimentin in ERK transport is not only to link ERK to the retrograde transport machinery. Through its binding to ERK, vimentin hides the phosphorylated ERK sites protecting them from phosphatase activity (Perlson et al., 2005; Perlson et al., 2006). Another possible method to avoid dephosphorylation is by hitchhiking in transport vesicles. Indeed, vesicles are used by toxins to avoid degradation (Pellizzari et al., 1999; Baldwin and Barbieri, 2009). This protection method is also used by JNK which is transported in axonal vesicles (Cavalli et al., 2005).

3.2.3. Neuronal response to injury and expression of regeneration associated genes (RAGs)

Although not all injury mechanisms are yet identified, an injury to the PNS triggers a robust response in the neuron cell body. Injury induces a transient increase in cAMP (Qiu et al., 2002) followed by expression and activation of several transcription factors, namely cAMP response element-binding protein (CREB) (Gao et al., 2004), c-Jun (Jenkins and Hunt, 1991; Leah et al., 1991), Elk-1 (Perlson et al., 2005), STAT-3 (Lee et al., 2004; Ben-Yaakov et al., 2012), activating transcription factor 3 (ATF-3) (Tsujino et al., 2000; Seijffers et al., 2006), sex determining region Y-box 11(Sox11) (Tanabe et al., 2003; Jankowski et al., 2006) and Mothers against decapentaplegic homolog 1 (Smad1) (Zou et al., 2009), which lead to a robust alteration of the transcription profile needed to increase the neuronal growth competence (Smith and Skene, 1997; Costigan et al., 2002; Xiao et al., 2002). Several RAGs have been identified as being expressed following injury. Among these genes are: cytoskeleton genes like tubulin and actin (Hoffman, 2010), Arginase-1 (Deng et al., 2009), neuropeptide-Y, vasointestine peptide (Vip) (Xiao et al., 2002), IL-6 (Cao et al., 2006), growth associated protein 43 (GAP-43) and CAP-23 (Bomze et al., 2001).

The changes induced by an injury can be seen *in vitro*. Naïve adult DRG neurons when cultured extend small, highly ramified neurites, while pre-injured DRG neurons extend long unbranched neurites. The transcription changes induced by an injury promote a transition from branching to elongation (Hu-Tsai

et al., 1994; Smith and Skene, 1997). One of the most striking features of injured DRG neurons is their ability to overcome myelin inhibition. Pre-injured DRG neurons are able to extend neurites even in the presence of an inhibitory environment, such as when plated on myelin (Qiu et al., 2002).

Several groups have tried to find a master regulator of the injury induced response. In this respect, cAMP was a major candidate for this role. Besides the increase of cAMP levels following injury, the fact that during development neurons maintain high levels of cAMP (Cai et al., 2001) led to the idea of cAMP as having a central role in the induction of axonal elongation. It was described that treatment with dibutyryl cyclic adenosine monophosphate (db-cAMP), a cell permeable analog of cAMP increased the regeneration ability of DRG neurons, even allowing to overcome myelin inhibition (Neumann et al., 2002; Qiu et al., 2002). However, cAMP induction does not reproduce the changes produced by a PNS injury, falling short to PNS injured neurons (Han et al., 2004; Blesch et al., 2012). Nevertheless, cAMP is still seen as one of the main targets to improve axonal regeneration. So far, manipulation of several of the RAGs led to improvements in axonal regeneration but none of the genes identified was able to mimic the changes produced by an injury (Blesch et al., 2012).

3.2.4. PNS regeneration: a robust but incomplete process

Following injury to the PNS, both motor and sensory neurons survive and are able to regrow past the injury site. Although the PNS is described as being able to fully regenerate, usually this regrowth does not lead to a complete functional recovery (de Ruiter et al., 2008; Hamilton et al., 2011). One of the main causes for the incomplete functional recovery is the misdirection of the regenerative axons. During regeneration of mixed nerves (composed of both sensory and motor tracts), injured axons are not able to find their previous targets, leading to an incorrect reinnervation. In practical terms, muscles can be reinnervated by motoneurons that previously were innervating a different muscle (English, 2005) or even by sensory neurons (Brushart et al., 2005). These abnormalities promote both motor deficits like contraction of muscles or synkinesis and disturbed sensory function (Dyck et al., 1988). Different surgical approaches have been tested unsuccessfully to improve correct axonal targeting (Evans et al., 1991; Bodine-Fowler et al., 1997; de Ruiter et al., 2008). The use of electrical

stimulation to improve targeting is controversial with beneficial (Brushart et al., 2005) or detrimental (Hamilton et al., 2011) outcomes. Furthermore, methods used to improve regeneration tend to increase axonal misdirection (English, 2005; Hamilton et al., 2011).

PNS regeneration is seen has a powerful process, particularly when compared to regeneration after a CNS injury. However this comparison can give the wrong impression that axonal regeneration in the PNS is a perfect process. We should not forget that although robust, PNS regeneration still has some drawbacks, namely the incorrect re-innervation.

4. CNS regeneration – why does it fail?

In contrast to the PNS, following injury CNS axons present limited ability to regenerate. This abortive regeneration translates in permanent sensory-motor impairments. Several differences have been pointed out to explain the lack of regenerative ability of CNS neurons, namely the inefficient Wallerian degeneration followed by the formation of the glial scar, that is a barrier to regenerating axons, and the limited neuronal cell-autonomous response to injury as CNS neurons are unable to activate many of the genes necessary for regeneration to take place.

In the following paragraphs the current view on the ineffective regeneration of the CNS will be explored.

4.1. Slow Wallerian degeneration and the formation of the glial scar

Support of CNS neurons is provided by oligodendrocytes and astrocytes. Oligodendrocytes provide myelin insulation to the CNS axons with a single cell being able to myelinate several axons, while astrocytes provide trophic support and are responsible for the homeostasis of the CNS. Following injury, oligodendrocyte apoptosis leads to an accumulation of myelin debris which together with astrocyte activation leads to the formation of a glial scar (Fig. 16). Furthermore, injury triggers a robust immune response with the production of cytokines and chemokines with coordinated infiltration of leukocytes.

Following injury, unlike Schwann cells, oligodendrocytes are not able to dedifferentiate and clear myelin debris. Indeed, loss of contact with axons and the robust inflammatory response leads to oligodendrocyte apoptosis (Vargas and Barres, 2007). Moreover, the blood-brain-barrier (BBB) is maintained following CNS injury, limiting the infiltration of circulating macrophages (George and Griffin, 1994; Popovich and Hickey, 2001) and the resident CNS microglia display limited ability to remove myelin when compared to infiltrating macrophages (Simard et al., 2006). The small infiltration of macrophages together with the lower ability of oligodendrocytes and microglia to phagocyte myelin leads to a slow, incomplete Wallerian degeneration, culminating in the accumulation of myelin debris.

Immediately following injury, astrocytes are activated and form a dense scar tissue surrounding the injured area (Silver and Miller, 2004). In this acute phase the main function of the activated astrocytes is to seal the injured area to prevent the injury area to extend (Rolls et al., 2009).

The glial scar is seen as detrimental for recovery following injury, since it impedes axonal regrowth (Fitch and Silver, 2008). Originally seen only as a physical barrier (Windle and Chambers, 1950), it was found that reactive astrocytes produce several extracellular matrix (ECM) proteins, namely members of the chondroitin sulfate proteoglycans (CSPG) that prevent axonal elongation (Verma et al., 2008), making the glial scar not only a physical, but also a biological barrier for regeneration. However it was found that the glial scar is important to restore the BBB and to separate the healthy tissue from the injured one, preventing damage progression to the uninjured tissue (Rolls et al., 2009). As such, damage control is made by sacrificing the possibility of regeneration of injured axons.

However, past the acute phase, further destruction of healthy tissue occurs during a process called secondary injury. The immune response plays an important role in this process, with neutrophil invasion of the lesion, followed by lymphocytes and later, macrophages. Later, there is an increase in TNF-α leading to the production of pro-inflammatory cytokines, promoting apoptosis of neuronal and glial cells and thereby increasing the damaged area (Lee et al., 2000; Genovese et al., 2008). From all infiltrating leukocytes, lymphocytes in particular are thought to promote damage during this secondary phase. In this

respect, mice lacking T lymphocytes were shown to present attenuated neuropathology after spinal cord injury (SCI) (Fee et al., 2003; Potas et al., 2006).

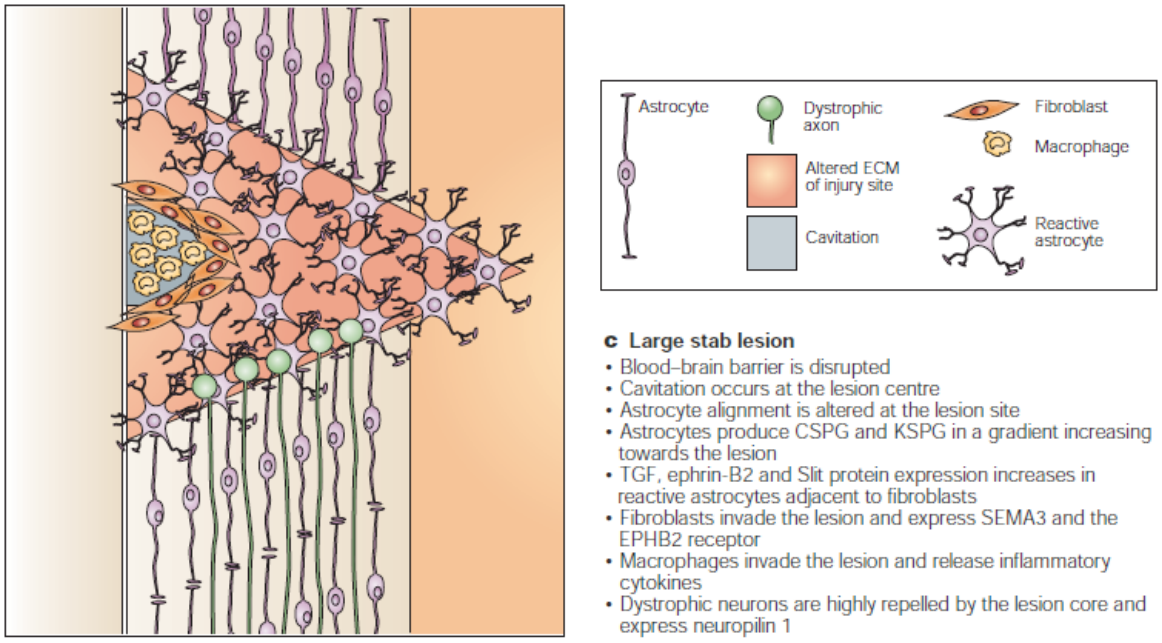

Figure 16. Composition and organization of the glial scar. Adapted from (Silver and Miller, 2004).

4.2. Inhibitory components are present in the CNS following injury

One of the major causes for the inability of CNS axons to regenerate is the harsh environment that is formed following injury. Along the formation of the glial scar there is an accumulation of myelin debris composed of several regeneration inhibitors. The inhibitory components that CNS axons face following injury can be divided in three categories: myelin associated inhibitors (MAI) (Lee and Zheng, 2012), canonical guidance molecules (Giger et al., 2010), and chondroitin sulfate proteoglycans (Galtrey and Fawcett, 2007) (Fig. 17). The relevance of the inhibitory environment in the CNS following lesion was shown in seminal studies, where axonal regeneration occurred through a peripheral nerve graft implanted following SCI (Richardson et al., 1980; David and Aguayo, 1981).

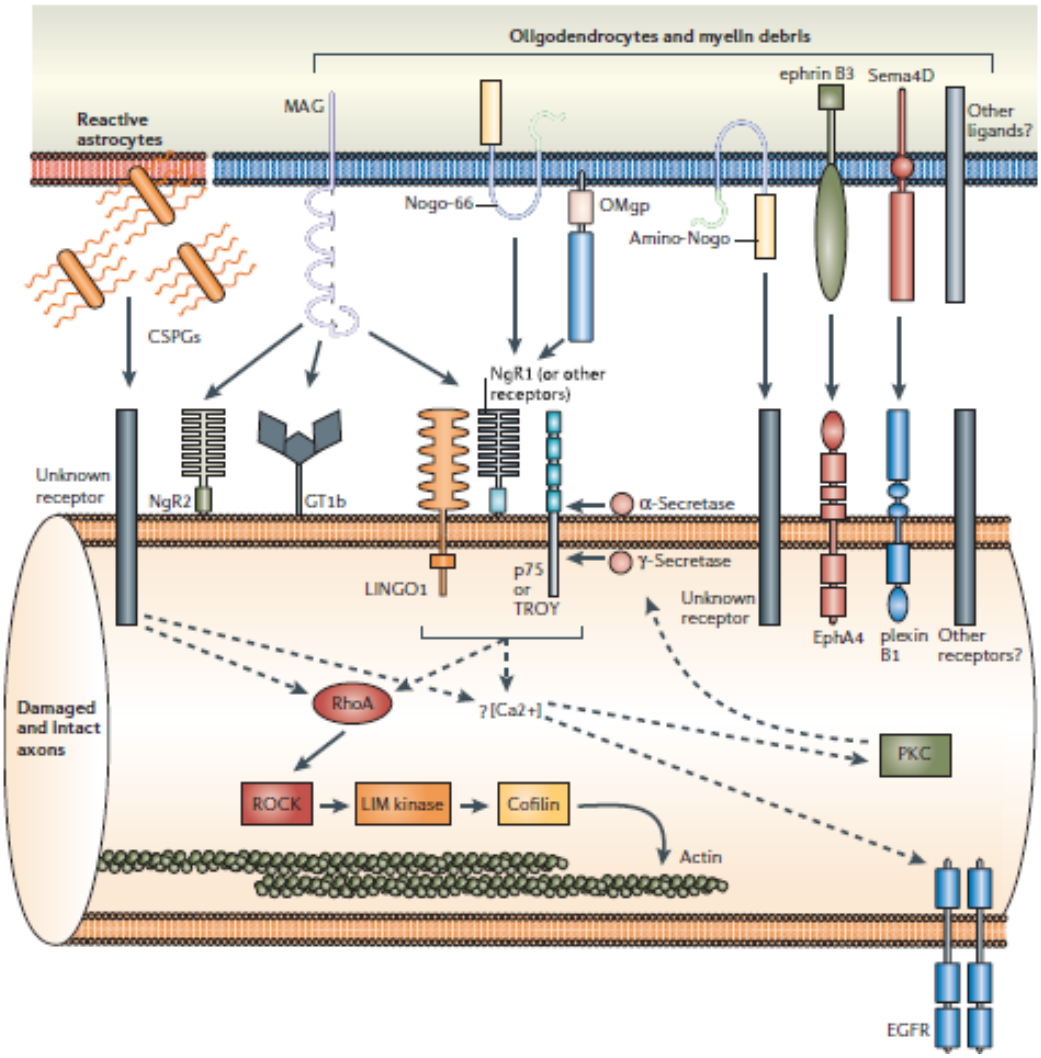

Figure 17. Axonal inhibition through exposure to inhibitory molecules of the glial scar. Injured CNS axons are exposed to multiple inhibitory molecules that signal through RhoA/Rho-associated kinase (ROCK). Adapted from (Yiu and He, 2006).

4.2.1. Myelin associated inhibitors (MAIs)

MAIs were the first inhibitory components of the CNS identified. CNS myelin was found to be inhibitory for neurite outgrowth (Schwab and Caroni, 1988). The production of an antibody against a myelin fraction (the IN-1 antibody) was able to alleviate the growth inhibition of myelin *in vitro* (Caroni and Schwab, 1988) and to promote corticospinal tract (CST) regeneration following SCI (Schnell and Schwab, 1990; Brosamle et al., 2000). Nogo was identified as being one of the myelin components responsible for this inhibition, as it was one of the targets of the IN-1 antibody (Chen et al., 2000; GrandPre et al., 2000). Three different isoforms of Nogo exist (Nogo-A, B and C) and Nogo possesses two main

inhibitory regions, a putative extracellular amino acid loop called Nogo-66, which is common to all isoforms, and a segment specific for Nogo-A (Oertle et al., 2003). Besides Nogo, MAG was also identified as a myelin inhibitor (McKerracher et al., 1994; Mukhopadhyay et al., 1994). MAG is the only inhibitor that is present both in PNS and in CNS myelin which could also account for the better regeneration of PNS axons. Although MAG is able to inhibit neurite outgrowth of several classes of neurons, it plays a dual role in axonal growth. Adult DRG neurons are inhibited by MAG, but DRG from newborn animals are stimulated by this protein. This behavior shift occurs within the first days of life (P4) and is thought to be related to the decrease in cAMP levels in neurons following maturation (Mukhopadhyay et al., 1994; DeBellard et al., 1996). More recently OMgp was also found to be another member of this family (Kottis et al., 2002; Wang et al., 2002b).

Extensive *in vitro* work led to the characterization of the molecular mechanisms by which MAIs promote growth cone collapse and outgrowth inhibition. The Nogo-66 receptor (NgR) was the first identified receptor for MAIs. It is a GPI-linked protein expressed in many types of neurons initially described as interacting with Nogo-66 (Fournier et al., 2001). Although the three known MAIs do not share any structural feature, MAG and OMgp are also able to interact with NgR (Domeniconi et al., 2002; Liu et al., 2002; Wang et al., 2002b). NgR lacks a cytosolic domain capable of transducing the inhibitory signals from MAIs and as such it needs a co-receptor capable of transducing the inhibitory signals. p75 was found to be a NgR co-receptor as neurons lacking p75 presented lower inhibition when facing MAIs (Wang et al., 2002a; Wong et al., 2002; Yamashita et al., 2002; Park et al., 2005; Shao et al., 2005). Nevertheless, p75 is not expressed in many neuron populations limiting its relevance in the *in vivo* inhibitory effect of MAIs. Later, Troy, another member of the tumor necrosis factor receptor (TNFR) family which is expressed in most of the adult neurons was identified as the co-receptor of NgR (Park et al., 2005; Shao et al., 2005). Furthermore, Lingo-1 was also found to be necessary for the complex NgR/p75 or NgR/Troy to be active and responsive to MAIs as neurons expressing a dominant negative Lingo-1 present lower responsiveness to MAIs (Mi et al., 2004). Although NgR was identified as being a MAI receptor mediating MAI inhibition, NgR KO mice are also inhibited by MAIs and do not present any improvement in CNS regeneration, suggesting the existence of other MAI receptors (Zheng et al., 2005). Indeed, PirB was later

identified as an alternative MAI receptor mediating neurite inhibition (Atwal et al., 2008), which might explain the lack of phenotype in NgR KO mice.

Myelin inhibition is mediated by the RhoA/Rho-associated kinase (ROCK) pathway (Lehmann et al., 1999) (Fig. 17). RhoA is a small GTPase that is maintained in the inactive form in the cytoplasm by Rho GDP dissociation inhibitor (RhoGDI) binding (Boulter et al., 2010). Upon MAI binding to p75, RhoGDI is displaced leading to RhoA activation (Yamashita and Tohyama, 2003). Active RhoA is able to activate its downstream effector ROCK leading to cofilin phosphorylation by Lim kinase culminating in growth cone collapse and axonal regeneration inhibition (Hsieh et al., 2006).

Based on the *in vitro* studies as well as on the promising results obtained with the IN-1 antibody to promote recovery following CNS injury, MAIs were an attractive therapeutic target to promote regeneration following CNS injury. However the use of genetic models lowered these expectations. OMgp and MAG KO mice do not present any increase in regeneration ability following SCI (Bartsch et al., 1995; Ji et al., 2008), while Nogo KO mice were reported to display different regeneration abilities, varying from extensive (Kim et al., 2003; Simonen et al., 2003) to none (Zheng et al., 2003; Lee et al., 2009). The lack of robust, consensual regeneration in MAI single KO mice, together with the fact that the three MAIs share the same mechanism of inhibition led to the idea that *in vivo*, Nogo, MAG and OMgp could play redundant roles in restricting axonal regeneration. However, the use of triple (MAG, OMgp and Nogo) KO mice did not clarify this question. In one of the studies using the triple KO, it was reported that indeed, knocking out MAIs generated a synergistic effect, increasing axonal regeneration following SCI (Cafferty et al., 2010). However, in another study, triple KO mice presented an increase in sprouting ability without showing any increase in axon regeneration (Lee et al., 2010).

Many of the myelin inhibitors signal through RhoA/ROCK. RhoA activity is blocked by the toxin C3-ADP-ribosyltransferase (C3) from Clostridium botulinum, while ROCK can be inhibited by Y27632. Although inhibition of RhoA/ROCK does not induce RAG expression in neurons, both C3 and Y27632 are able to block myelin inhibition (Dergham et al., 2002; Winton et al., 2002) and its use *in vivo* promotes axonal regeneration following spinal cord injury (Dergham et al., 2002; Boato et al., 2010).

MAIs are by far the most studied inhibitors in the CNS. However, the existence of numerous inhibitors besides MAIs may underlie the successive flops obtained with MAI KO mice.

4.2.2. Guidance cues

Guidance molecules are of extreme importance during development for the correct pathfind of axons. Their expression in the adult CNS indicates that they may play other roles like the maintenance of the established network. However their role following CNS injury is not well studied. Among the most studied guidance molecules are Semaphorins, Ephrins and Repulsive Guidance Molecules (RGM).

4.2.2.1.Semaphorins

Semaphorins are a vast family of proteins that are important for axonal guidance by interacting with plexin receptors. Class 3 semaphorins (SEMA-3) are the only ones secreted and require neuropilin as a co-receptor (Yoshida, 2012). SEMA-3 is the most studied in the context of axonal regeneration. *In vitro* it leads to growth cone collapse of both embryonic and adult DRG neurons (Reza et al., 1999). *In vivo*, following injury, SEMA-3 expression is increased by fibroblasts present in the glial scar. This expression forms an exclusion zone for regenerating axons (Pasterkamp et al., 1999; De Winter et al., 2002). The use of SM-216289, an agent that diminishes the effects of SEMA-3 by blocking its interaction to neuropilin-1/plexin A (Kikuchi et al., 2003) leads to an increase in regeneration of serotonergic fibers following injury, but is unable to increase CST regeneration (Kaneko et al., 2006). Besides SEMA-3, other semaphorins have been linked to inhibition following CNS injury, namely semaphorin 4D (Moreau-Fauvarque et al., 2003), semaphorin 7A (Pasterkamp et al., 2007) and semaphorin 6B (Kury et al., 2004).

4.2.2.2. Ephrins

The exposure of neurons to ephrins leads to axonal repulsion. Ephrin B3 is present in CNS myelin while Ephrin B2 is expressed in reactive astrocytes following injury (Bundesen et al., 2003; Benson et al., 2005). Both signal

inhibition through the EphA4 receptor and lead to neurite growth inhibition (Benson et al., 2005; Fabes et al., 2006). *In vivo*, blockage of Ephrin A4 receptor does not promote regeneration, but CST sprouting is observed (Fabes et al., 2007).

Besides axonal growth inhibition, ephrins may be important in the glial scar formation. This hypothesis is supported by the fact that the EphA3 receptor and EphA7 are expressed in astrocytes following injury (Irizarry-Ramirez et al., 2005; Figueroa et al., 2006) and also by the reduced glial scar formation in Ephrin A4 receptor KO mice (Goldshmit et al., 2004).

4.2.2.3. Repulsive guidance molecules (RGM)

RGM are a family of cell membrane associated glycosylphosphatidylinositol anchored glycoproteins. This family was identified as being repulsive and, important for the guidance of chicken temporal retinal axons (Monnier et al., 2002). In mice, 3 RGM have been identified (RGMa, RGMb and RGMc). Following SCI, there is an increased expression of RGM around the injured area leading to axon growth inhibition (Schwab et al., 2005; Hata et al., 2006). Local neutralization of RGMa with a specific antibody promotes axonal regeneration and functional recovery (Hata et al., 2006). Neogenin receptor was identified as being the only receptor for RGMa, and it mediates its inhibitory effect (Rajagopalan et al., 2004) by a mechanism dependent on the RhoA/ROCK pathway (Conrad et al., 2007). Besides axonal guidance and inhibition several other functions were described for RGMa such as importance in neural tube morphogenesis, cell adhesion, cell migration, cell polarity and cell differentiation (Key and Lah, 2012).

4.2.3. Chondroitin sulphate proteoglycans (CSPGs)

Upon injury, astrocytes are activated and start the production of extracellular matrix proteins, the CSPGs. These proteins represent a vast family of ECM proteins composed of a protein core linked to sulphated glycosaminoglycan chains. Some members of this family are: aggrecan, brevican, phosphacan, neurocan, versican and NG-2 (Silver and Miller, 2004; Rolls et al., 2009). Their importance as inhibitors of axonal regeneration is supported by three evidences:

following lesion there is an increase in the production and secretion of CSPGs (McKeon et al., 1999; Jones et al., 2003; Tang et al., 2003); *in vitro* assays show that CPSGs inhibit neurite outgrowth by growth cone collapse (Dou and Levine, 1994; Friedlander et al., 1994; Milev et al., 1994; Yamada et al., 1997; Schmalfeldt et al., 2000) and treatment with chondroitinase, an enzyme that removes glycosaminoglycans from CSPGs (Prabhakar et al., 2005), is able to decrease the inhibitory environment formed in the glial scar and promote axonal regeneration following SCI (Bradbury et al., 2002; Yick et al., 2003; Caggiano et al., 2005). CSPGs mediate its inhibitory effect through receptor protein tyrosine phosphatases (Aricescu et al., 2002). Recently, NgR was also found to be a receptor for CSPGs, providing evidence that CSPG may share the molecular mechanism of inhibition with that of MAIs (Dickendesher et al., 2012).

4.3. CNS neurons are not able to increase their intrinsic ability to regenerate

In contrast to PNS axons, CNS neurons are not able to respond to injury (Zurn and Bandtlow, 2006; Huebner and Strittmatter, 2009). Most CNS neurons fail, or have a mild increase in the expression of RAGs following injury (Fernandes et al., 1999; Ylera et al., 2009). This constitutes a major drawback in axonal regeneration. In fact, when RAGs are overexpressed in CNS neurons, increased regeneration ability through an inhibitory environment is achieved (Bomze et al., 2001; Kwon et al., 2007; Yang et al., 2012).

Although some of the molecular mechanisms underlying RAG activation following PNS lesion are known, the reasons underlying the inability of CNS neurons to increase RAG expression were not addressed yet. In fact, the injury signals in the CNS that could prompt neurons to a pro-regenerative status have not yet been identified. Adult CNS neurons are maintained in a non-regenerative mode due to suppression of mTOR by PTEN (Park et al., 2008) and by the activity of cytokine signaling 3 (SOCS-3), an inhibitor of the JAK-STAT-3 pathway (Smith et al., 2009). Following injury, PTEN and SOCS-3 activity is maintained preventing any increase in the intrinsic ability of CNS axons to regenerate. Accordingly, it was shown that PTEN or SOCS-3 deletion improves regeneration of CNS neurons by increasing their growth capacity (Park et al., 2008; Smith et al., 2009).

Moreover, these 2 proteins function in independent mechanisms since deleting both further improves axonal regeneration following injury (Sun et al., 2011).

Recently, in *C. elegans*, the conserved Arf guanine nucleotide exchange factor EFA-6, was also reported to be an intrinsic inhibitor of regrowth that operates by affecting axonal microtubule dynamics, acting downstream of and/or in parallel with DLK (Chen et al., 2011) [58].

This evidence suggests that besides extrinsic factors, CNS axonal regeneration is also impaired by intrinsic repressors. As such, the manipulation of the intrinsic control of regeneration has emerged recently as a good target to promote axonal regeneration.

5. Conditioning injury model

Despite the general inability of CNS axons to regenerate, CNS axonal regeneration is possible: DRG neurons possess a peripheral branch that regenerates when injured (Fig. 18*A*), and a central branch that enters the spinal cord originating the dorsal column fibers that does not regenerate upon injury (Fig. 18*B*). However, when the peripheral branch is lesioned approximately 1 week prior to the lesion to its central branch (known as conditioning lesion), the central axons are prompted to regenerate and are able to overcome the glial scar inhibitory effect, regenerating to a significant extent (Richardson and Issa, 1984; Neumann and Woolf, 1999) (Fig. 18*C*). Besides the increase in regeneration of dorsal column fibers of conditioned DRG neurons *in vivo*, the consequences of a conditioning injury can also be observed *in vitro*, as conditioned DRG neurons have an increased neurite outgrowth ability (Fig. 18*D*,*E*) and are able to grow on a myelin substrate (Qiu et al., 2002).

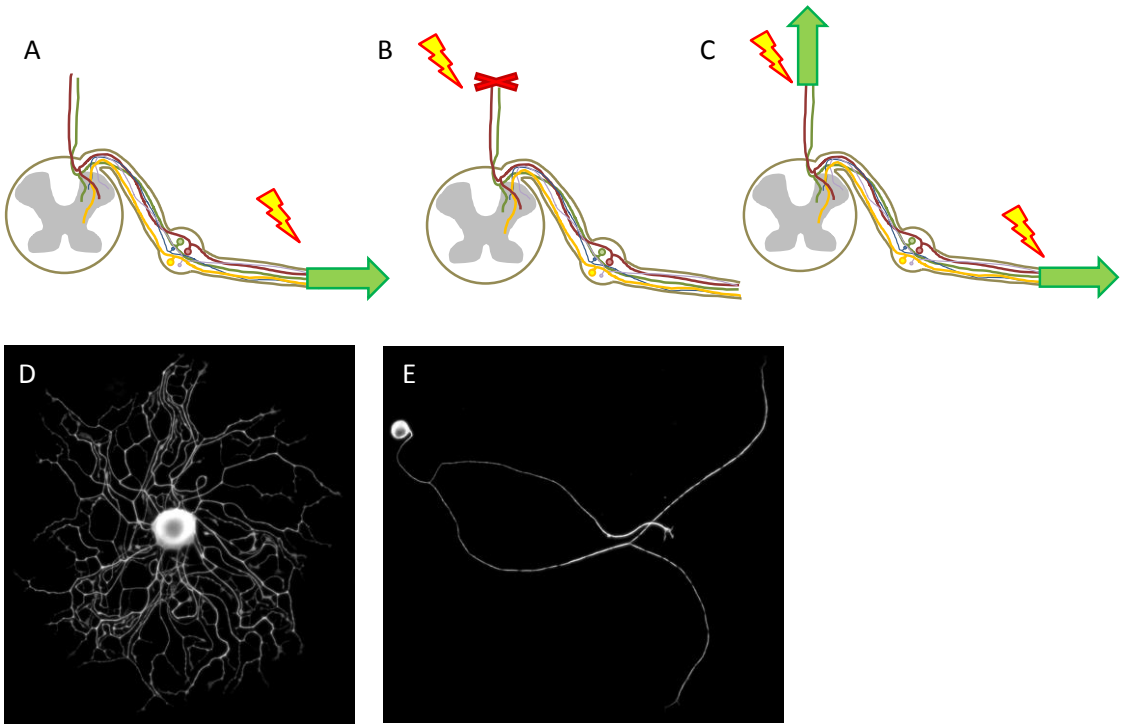

Figure 18. The conditioning effect. The peripheral branch of DRG neurons is able to regenerate when injured (A), while the central one is not (B). However when an injury to the peripheral branch is performed before the injury to the central branch, the central branch can now regenerate (C). This result can also be seen *in vitro*, as naïve DRG neurons extend small branched neurites (D), while conditioned DRG neurons extend long unbranched neurites (E).

The conditioning effect is the consequence of the activation of the regenerative machinery prior to the CNS lesion. The increase in the regeneration ability of DRG neurons encompasses RAG expression (Smith and Skene, 1997; Costigan et al., 2002) and changes in axonal transport induced by the PNS lesion (Hoffman, 1989; Hoffman and Luduena, 1996). In fact, the response starts as soon as one day following injury (Qiu et al., 2002), and has a long-lasting effect, since RAGs are expressed as long as two months following the conditioning injury (Ylera et al., 2009).

The greatest regenerative effect is obtained when the conditioning injury is performed one week before the central injury. If the conditioning injury is performed following SCI, it does not provide any improvement in regeneration (Neumann and Woolf, 1999). A recent study showed that, although it does not improve axonal regeneration, the conditioning injury performed after SCI also increases the intrinsic ability of DRG neurons to regenerate (Ylera et al., 2009). The inability of a “post conditioning” injury to promote regeneration is due to the

establishment of a thick glial scar that does not allow the regrowth of axons, even when expressing RAGs. In fact, the authors elegantly show that when performing laser single axon lesion, that cause minimal scarring, a conditioning lesion performed two weeks after is able to promote the regeneration of the central axons (Ylera et al., 2009). These experiments suggest that following a SCI, there is a small window of opportunity for treatment, before the glial scar becomes too thick. A prolonged delay in treatment, allows the formation of a thick glial scar that prevents the regeneration of central axons, even when these have an increased intrinsic ability to regrow.

The conditioning effect represents a good model on how increasing the intrinsic ability of axons to regrow can improve axonal regeneration. As such, understanding the mechanism by which a conditioning injury prompts CNS axons to regenerate has been the focus of attention on the axonal regeneration field for many years. The most relevant finding was the fact that at least in part, the conditioning effect was mediated by cAMP (Qiu et al., 2002). This finding together with others made cAMP the main target to promote regeneration. In fact, the use of phosphodiesterase (enzyme responsible for cAMP degradation) inhibitors, such as rolipram was shown to increase cAMP levels and improve recovery following SCI (Nikulina et al., 2004; Pearse et al., 2004). However, the use of phosphodiesterase inhibitors leads to disabling nausea, limiting its clinical application.

Several studies using array techniques identified broad changes in gene expression of conditioned neurons (Costigan et al., 2002; Cao et al., 2006). In fact these changes are regulated by the activation of multiple transcription factors including c-Jun, sox11, CREB, Smad1, ATF-3, AKRD1, nuclear factor interleukin 3 regulated (NFIL3) and STAT-3 (Kiryu-Seo and Kiyama, 2011). The conditioning injury triggers the expression of traditional RAGs such as GAP-43 and CAP-23 (Hoffman, 1989), but it also allowed the identification of novel RAGs such as Arg-1 (Cai et al., 2002; Deng et al., 2009), leukemia inhibitory factor (Cafferty et al., 2001), IL-6 (Cafferty et al., 2004; Cao et al., 2006) and tissue plasminogen activator (Minor et al., 2009). However, none of the identified transcription factor or RAGs is able to reproduce the extension of the conditioning effect. As such, the quest for clinically relevant molecules that mimic the conditioning lesion should be pursued.

Besides the transcription changes observed following conditioning injury, another important alteration occurs that may contribute to axonal regeneration. It has been shown that a conditioning injury is able to increase axonal transport in both DRG branches (Hoffman and Luduena, 1996; Hoffman, 2010). Furthermore, these alterations were identified as being independent of cAMP, since cAMP is able to trigger transcription changes but it does not alter axonal transport (Han et al., 2004). It has also been shown that conditioned neurons present increased protein synthesis in their axons following injury, and this local synthesis may also underlie their high ability to regenerate (Zheng et al., 2001; Verma et al., 2005).

Recently, histone deacetylase 5 (HDAC5) has been described as a key element in the conditioning response. Upon injury, the initial depolarization wave is able to activate protein kinase Cμ at the cell body promoting nuclear export of HDAC5. Absence of HDAC5 at the nucleus leads to increase histone acetylation activating the pro-regenerative program (Cho et al., 2013). This epigenetic mechanism controls the switch from non-regenerative to growth-competent axons. This initial phase where histones are acetylated prime the neuronal DNA to the positive injury signals that will be conveyed later through the retrograde transport machinery (Cho et al., 2013). In the proposed mechanism, the initial depolarization alters neurons so that they can respond rapidly to further signals, explaining why the initial depolarization by itself cannot trigger a long-lasting response.

In summary a conditioning injury triggers a robust response to induce regeneration. Transcriptional changes together with changes in local protein synthesis and axonal transport make the conditioning injury a powerful paradigm that can hardly be reproduced by the stimulation of a single gene/protein.

6. Important tools to study CNS axonal regeneration

The complexity of the CNS makes the study of CNS axonal regeneration a great challenge. It is important to distinguish the different types of axonal regeneration, as well as the different models available to study it. In this chapter such features will be discussed.

6.1. Axonal plasticity: regeneration vs sprouting

The classification of axonal growth following injury is a matter of debate in the regeneration field. Indeed in many studies, the terms regeneration and sprouting are incorrectly used as synonyms. Although both types of axonal growth are induced by injury and can lead to functional improvements, they represent distinct types of growth. Axonal regeneration consists on the regrowth of an injured axon through and beyond the lesion site (Fig. 19*A*). This type of growth is very characteristic of PNS axons, while in the CNS it is very limited (Lee and Zheng, 2012; Tuszynski and Steward, 2012). Sprouting accounts for the capacity of axons to grow new small branches to establish new connections. This phenomenon can happen both in injured and non-injured (spared) axons. In the proximal side of an injured axon, when it faces a high inhibition in the injury site and cannot regrow beyond it, it may form a small branch that is able to establish a new connection with an uninjured neuron (Fig. 19*B*). This new connection may allow the indirect reconnection of the injured axon to its former target. This kind of regrowth is known as regenerative sprouting (Tuszynski and Steward, 2012). Injury can also induce sprouting of spared axons. In this case, spared axons are able to establish new connections that can reinnervate the targets that lost their connections (Fig. 19*C*). This process is known as collateral sprouting (Lee and Zheng, 2012).

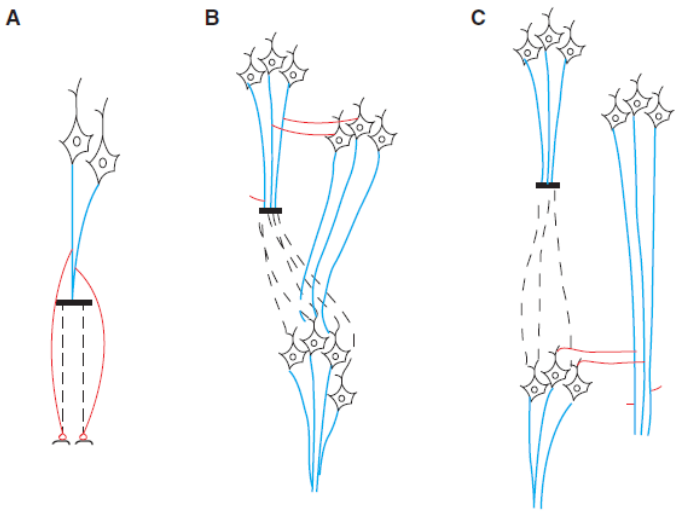

Figure 19. Axonal regeneration and sprouting. Injured axons can regenerate through the glial scar (A), or extend lateral sprouting to form new connections (B). Spared axons may also form sprouts that may reinnervate axons that lost their connections (C). Adapted from (Giger et al., 2010).

If the correct models and methodology are not used, the distinction between regeneration and sprouting becomes challenging to do. If some axons are inadvertently spared and they are able to sprout, this may lead to the wrong notion that an increased regeneration has taken place.

Regeneration through long distances in the CNS is a daunting task. Not only the environment of the CNS is not favorable, but also CNS neurons do not have the proper tools for such a long regrowth. As such, sprouting is probably more promising as a mechanism to improve functional recovery.

6.2. Injury models to study CNS axonal regeneration.

The spinal cord is composed of several axonal tracts with different functions and regeneration abilities. In this chapter I will discuss the different tracts that can be used to study axonal regeneration as well as their main advantages /disadvantages.

6.2.1. Dorsal column fibers

The dorsal column fibers are ascending sensory fibers. They originate in the dorsal root ganglia where their cell body is. These are pseudo-unipolar neurons with one axon going to the PNS and the other ascending through the dorsal white matter in the spinal cord (Fig. 20) to the nucleus gracilis in the brainstem (Hebel and Stromberg, 1986). When a dorsal hemisection is performed, all dorsal column fibers are sectioned. The fibers can be labeled by a tracer injection like cholera toxin-β (CT-β) in the sciatic nerve. This constitutes a simple model to study the mechanisms underlying CNS regeneration and to test therapies to enhance axonal regeneration (Tuszynski and Steward, 2012).

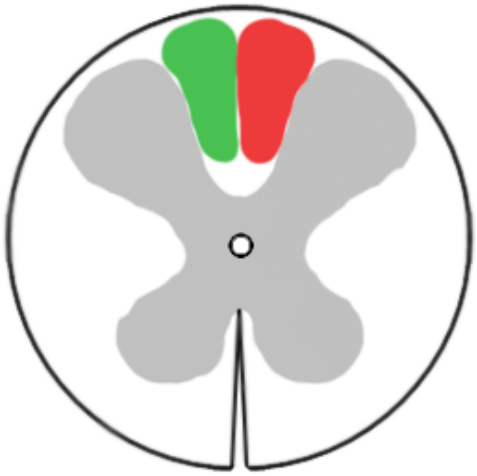

Figure 20. Dorsal column fibers. Dorsal column fibers (in the red and green areas) ascend in the dorsal white matter.

6.2.2. Raphespinal fibers

The raphespinal tract originates in the brainstem raphe nuclei and is composed of motor descending fibers. They descend through the spinal cord as dispersed bundles of axons surrounding the central gray matter (Fig. 21). These fibers are one of the most plastic fibers in the CNS with high ability to regenerate. Due to their sparse localization within the spinal cord, only a complete transection is able to injure all raphespinal fibers. These are the only serotonergic fibers present in the spinal cord. As such an immunohistochemistry to serotonin (antibody against 5-hydroxytyptamine-5HT) labels the raphespinal fibers in the spinal cord (Lee et al., 2010). Several studies reported improvements in raphespinal fiber regeneration using models of incomplete injury, however such models do not exclude the possibility that the increased fiber number below the injury site is due to sprouting of spared fibers. The correct conclusion that applies to this case is that an increase in axonal growth was found instead of an increase in axonal regeneration (Cafferty et al., 2010; Hellal et al., 2011).

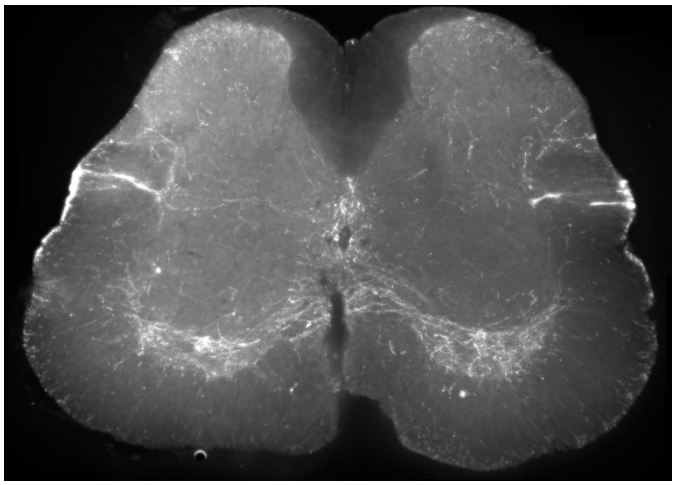

Figure 21. Raphespinal fibers. The raphespinal fibers descend in the spinal cord as dispersed bundles of fibers in the gray matter. Lumbar spinal cord cross section immunostained for 5HT.

6.2.3. Rubrospinal fibers

The rubrospinal fibers originate in the red nuclei of the midbrain and are composed of motor fibers that descend through the dorsal part of the lateral column. Their regeneration can be studied with a model of lateral hemisection by labeling the fibers with tracer injection in the brainstem. But again, if only a lateral hemisection is performed, the possibility of sprouting of spared axons cannot be excluded. This tract is much more prone to regenerate than the other motor tract, the CST. However the fact that in humans it is residual makes it unattractive to study (Tuszynski and Steward, 2012).

6.2.4. Corticospinal tract (CST)

The CST is the most important tract for voluntary motor function. It originates in the sensory motor cortex and in rodents it descends mainly in the dorsal white matter (Fig. 22), with a small number of fibers descending through the lateral white matter. In humans, the lateral tract is the major CST tract. The CST is one of the most refractory tracts to regeneration. It can be labeled by tracer injection, like biotin dextran amine in the layer 5 of the sensorimotor cortex. Usually, the dorsal hemisection is used as a model in rodents since it injures almost all axons. Nevertheless the sparing of the lateral fibers cannot be excluded in this model. Due to its lower ability to regenerate, the use of a complete transection, where the glial scar formed is much bigger than after a hemisection, and where a gap of

millimeters may occur, is not advisable. There are no studies showing convincingly that there is CST regeneration following a complete transection. A contusion injury constitutes an alternative to the complete injury, since the tissue retraction is avoided. However even using a severe contusion, some CST fibers are spared (Tuszynski and Steward, 2012).

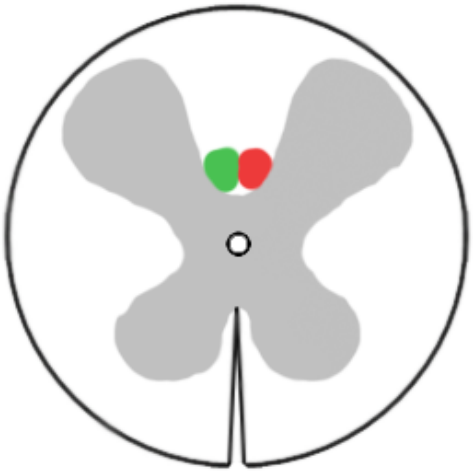

Figure 22. Corticospinal tract. The corticospinal fibers (in the red and green areas) descend in the dorsal white matter close to the gray matter.

6.2.5. Optic nerve

Although it is not a spinal cord tract, the optic nerve is one of the most used CNS tracts to study both regeneration (Park et al., 2008; Smith et al., 2009) and guidance/elongation during development (Mann and Holt, 2001; van Horck et al., 2004; Jung et al., 2012). The retinal ganglion cells are located in the inner surface of the retina and their axons form the optic nerve. The optic nerve has a simple structure with an easy access to perform crush and where regenerating axons can be labeled by intravitreal injection of a tracer. These characteristics make it a good and easy model to study axonal regeneration in the CNS. Moreover, with the increased use of transgenic mice, it became an even more powerful tool. Lentivirus expressing Cre recombinase can be injected intravitreally in animals with floxed genes. This model constitutes an easy, fast and economic way of generating conditional/tissue specific KO mice that can be used to study the importance of specific genes in axonal regeneration (Park et al., 2008; Smith et al., 2009; de Lima et al., 2012).

6.3. *In vivo* imaging, a new tool to study axonal regeneration

Classical tools for the analysis of axonal regeneration in spinal cord injury models consist in the labeling of a particular axonal tract followed by measuring the extension of axonal regrowth through or beyond the injury site. Despite that this approach led to important achievements, it has several limitations. Although time course experiments can be performed to analyze axonal regeneration, this leads to several static pictures at different time points which may not allow the visualization and comprehension of the dynamic response to injury, and the regrowth process. Another important limitation is the possible variability of neuronal tracing due to variance in the amount and site of tracer injection. If not performed correctly, it may also lead to the labeling of undesired fibers.

The inability to surpass these classical tools was due to technical limitations, but also to the absence of suitable mouse models. Recently several mouse models have been generated for use in *in vivo* imaging. These models consist in the expression of a fluorescent protein in a subset of neurons, usually in less than 10% of the neurons (Feng et al., 2000; Kerschensteiner et al., 2005; Young et al., 2008). Genetically labeled neurons also present higher expression than neurons infected with viruses. With the correct mouse model, superficial axons in the spinal cord, like dorsal column fibers, can be imaged with a basic epifluorescent microscope. The main advantage of *in vivo* imaging when compared to classical methods is the possibility of the direct observation of the response to injury. It also allows a clear distinction between regenerative and spared axons, or between inhibition of degeneration and increase in regeneration. In short it allows unraveling the mechanisms by which axons respond to injury (Laskowski and Bradke, 2012).

Although powerful, *in vivo* imaging is still a recent technique with many limitations. It requires extensive expertise both in spinal cord surgery and imaging. The prolonged anesthesia together with the extensive and multiple surgeries performed increases the risk of complications like infection. Also, long term observations are even more demanding due to the formation of a thick glial scar in the injury site. Another difficult task is the choice of good landmarks like blood vessels that allow for the correct measurement of the axonal regeneration distance. Even with state of the art multiphoton microscopy, live imaging allows

only observation of relatively superficial axons (50μm deep), limiting the axonal tracts evaluated.

Recently two methods were described for chronic imaging of spinal cord injury. They consist in the implantation of a homemade chamber in the vertebral column (Farrar et al., 2012) or of a glass window attached to the spinal cord (Fenrich et al., 2012). Both methods present minimal scarring enabling successful imaging for as much as 22 imaging sessions up to 350 days following surgery. The other main advantage is the possibility of visualizing chronic injury with a single surgery. It is exciting to see if the next developments of this technique fulfill its great potential.

7. Possible treatments to achieve axonal regeneration following spinal cord injury.

In the last two decades, methylprednisolone was the only drug administered following SCI. Although initially described as improving functional recovery, in recent studies its benefits have been questioned (Coleman et al., 2000; Hurlbert, 2000; Bracken, 2001; Miller, 2008), which led to abandon methylprednisolone as a treatment to SCI. Nowadays the only treatment available following SCI is rehabilitation. The main goal of rehabilitation is to prevent further complications and maximize physical function. As such, there is a total absence of therapies to promote axonal regeneration and functional recovery of patients with acute or chronic SCI.

The limitations for axonal regeneration in the CNS are well identified. The lack of an intrinsic regenerative ability, together with the formation of a thick glial scar, where injured axons are exposed to several inhibitors poses a great challenge to achieve axonal regeneration. As such, most pre-clinical strategies consist in overcoming these two main obstacles, either by stimulating neurons to increase the intrinsic ability to regrow their axons or by modulating the injury site environment so that it becomes less inhibitory.

7.1. Modulation of the injury site environment

Modulation of the injury environment was the first strategy used successfully in animal models. The neutralization of the inhibitors present in the spinal cord following injury is an attractive strategy for treatment. Initial studies using IN-1, an antibody produced against myelin components showed promising results, promoting axonal regeneration following SCI (Schnell and Schwab, 1990). As described above, IN-1 recognizes Nogo-A, the most studied regeneration inhibitor (Chen et al., 2000), but it is not specific for Nogo-A (Caroni and Schwab, 1988). Other antibodies produced against Nogo showed less pronounced improvements (Liebscher et al., 2005). Even when neutralizing Nogo, several other inhibitors are still present. Many of the inhibitors like other MAIs or CSPG share a common receptor, NgR. The blockage of NgR should then have a broader effect since it may block the effect of several inhibitors. Indeed peptide blocking of NgR was reported to improve axonal regeneration (GrandPre et al., 2002; Li and Strittmatter, 2003; Cao et al., 2008). However, the use of the blocking peptide was re-assessed and no relevant improvements were found (Steward et al., 2008). Besides Nogo, antibody blocking of other inhibitors like RGMa (Hata et al., 2006) and proteoglycan NG2 (Tan et al., 2006) also diminishes the inhibitory environment following injury promoting axonal regeneration.

CSPGs are one of the most important inhibitory components of the glial scar. The degradation of the glycosaminoglycan chains by chondroitinase ABC diminishes the inhibitory effect of CSPGs and its use *in vivo* has led to significant functional improvements in CNS regeneration (Moon et al., 2001; Bradbury et al., 2002; Yick et al., 2003; Caggiano et al., 2005; Tester and Howland, 2008). Although blocking a particular inhibitor may lead to improvements, the reality is that several other inhibitory molecules are still present and may take the place of the inhibitor neutralized. Multi-targeting several inhibitory classes should promote better outcomes.

Another important approach to overcome the inhibitory environment at the lesion site is to provide “bridges” that provide axons an alternative path without contacting the inhibitors. The first bridge successfully used was a peripheral nerve graft which allowed for regeneration of CNS axons (Richardson et al., 1980; David and Aguayo, 1981). Transplant of fetal spinal cord to the injury site also promotes the growth of axons within the injury site (Jakeman and Reier, 1991).

The use of cell transplantation has caught most of the attention in an attempt to "build bridges" in the injury site. The main goal is to have cells that integrate well within the injury and form a cellular matrix capable of linking the rostral and distal injury borders (Lu and Tuszynski, 2008). The successful use of PNS graft by (David and Aguayo, 1981), raised the possibility that Schwann cells could ameliorate the environment following SCI improving functional recovery. Indeed it has been shown that Schwann cell transplantation produces functional recovery (Pinzon et al., 2001; Takami et al., 2002). In fact, the studies with cell transplantation following SCI to improve recovery have been growing extensively. In this respect, bone marrow stromal cell (MSC) transplantation has been widely used to facilitate the bridging following injury. When used in combination with other treatments that increase the intrinsic regeneration ability of neurons, MSCs have a synergistic effect (Lu et al., 2004; Kadoya et al., 2009). Transplantation of olfactory ensheating cells (OEC) has also been reported to promote improvements following SCI. It has been shown that OEC transplantation improves both survival and regeneration following SCI by producing neurotrophic factors, as well as by reducing the CSPG production by astrocytes (Tohda and Kuboyama, 2011). One of the most attractive features of MSC and OEC transplantation is the possibility of autologous transplant. Besides trophic support, cell transplantation can be used to replace the lost cells following injury. In this case, stem cell transplantation becomes important in doing so. Both embryonic stem cells (ESCs) and neural stem cells (NSCs) have been used successfully, improving functional recovery and being able to differentiate in oligodendrocytes and neurons (Pego et al., 2012).

Recently, the implantation of embryonic neural stem cells following spinal cord injury has been shown to increase regeneration and functional recovery remarkably. The implanted cells were able to extend axons and build new circuits that led to improved function (Lu et al., 2012).

7.2. Increase of the intrinsic regeneration ability of CNS neurons

cAMP is one of the most important intracellular signals. It is synthesized from ATP by adenylyl cyclase at the plasma membrane and it is degraded by phosphodiesterase. For the past 15 years, cAMP got most of the attention in the axonal growth and regeneration field. Young CNS neurons have high ability to

elongate. This ability is lost during development due to a decrease in cAMP levels (Cai et al., 2001). The high levels of cAMP during development also make axon growth cones attracted to guidance cues like netrin-1 and SEMA-3, that are repellent to adult neurons (Ming et al., 1997; Song et al., 1998). Two of the most relevant findings were that cAMP can prompt neurons to overcome myelin inhibition *in vitro* (Cai et al., 1999; Qiu et al., 2002), and that treatment with db-cAMP, a cell permeable analog of cAMP increases the regeneration of dorsal column fibers following SCI (Neumann et al., 2002; Qiu et al., 2002). This led to the notion that the manipulation of cAMP levels in neurons could allow them to overcome the inhibitory environment following SCI. The use of phosphodiesterase inhibitor drugs like rolipram increases cAMP levels by preventing its degradation (Udina et al., 2010), leading to increased axonal regeneration and functional recovery (Nikulina et al., 2004; Bretzner et al., 2010). However, the use of such drugs induces disabling nausea making them unsuitable for treatment. Furthermore, the robust effects of cAMP initially reported have been questioned by recent studies (Han et al., 2004; Blesch et al., 2012).

Besides cAMP, some of its downstream targets such as Arg-1 or IL-6 have been tested as possible enhancers. IL-6 was identified as being able to induce regeneration (Cafferty et al., 2004; Cao et al., 2006) and daidzein (a soy isoflavone) has been identified as a potent inducer of Arg-1 expression that can induce axonal protection and growth (Ma et al., 2010). However, none of them has shown enough potential to be relevant in an *in vivo* human condition.

Recently, the regeneration field has been focused on PTEN and SOCS-3. These 2 proteins were identified as potent regeneration inhibitors, as their removal promotes a robust increase in regeneration (Park et al., 2008; Sun et al., 2011; de Lima et al., 2012). Despite the robust effects described so far, further studies are needed to strengthen their role in axonal regeneration.

Neurotrophins are a class of small molecules that are important for the regulation of neuronal functions. NGF was the first neurotrophin identified. After NGF, many others were identified: BDNF, neurotrophin 3 and 4 (NT-3/4) and GDNF. They regulate neuronal survival and the interaction of neurons with their targets (Kirstein and Farinas, 2002). Moreover, they are also capable of inducing axonal growth and RAG expression (Gillespie, 2003) and to suppress myelin inhibition by increasing cAMP levels (Cai et al., 1999; Gao et al., 2003). When

applied following SCI, neurotrophins lead to functional improvements and to axonal regeneration of different tracts. NGF, BDNF and NT-3 are the most used ones with reports of all of them showing increased axonal regeneration (Schnell et al., 1994; Oudega and Hagg, 1996; Bregman et al., 1997; Bradbury et al., 1999; Oudega and Hagg, 1999; Song et al., 2008). The injection of lentivirus expressing NT-3 distally to the injury site was shown to create a chemoattractant gradient allowing axonal bridging of the injury site (Taylor et al., 2006).

Inflammation is a crucial step following injury encompassing the production of pro-inflammatory cytokines like TNF-α and IL-1β, and leukocyte recruitment. Usually its effects are seen as deleterious, and as discussed above, methylprednisolone a potent anti-inflammatory drug was previously seen as the only treatment available. Although it was reported initially as improving functional recovery, later studies questioned such evidences. The use of the anti-inflammatory cytokines interleukin 10 (IL-10) and IL-6 has shown improvements in recovery following SCI. IL-10 has a neuroprotective effect and its use promotes neuronal survival of injured neurons (Zhou et al., 2009), while IL-6 increases the axonal growth ability of neurons and overcomes myelin inhibition (Cafferty et al., 2004; Cao et al., 2006). Its use following injury promotes functional recovery by increasing axonal regeneration (Cafferty et al., 2004; Cao et al., 2006; Yang et al., 2012). Another evidence that the inflammatory response could be modulated to improve axonal regeneration is the ability of activated macrophages to promote regeneration of retinal ganglion cells following optic nerve injury. Activated macrophages are able to produce oncomodulin that increases the ability of neurons to grow their axons (Yin et al., 2006). However, in the context of SCI, the use of zymosan, a yeast cell wall preparation known for its ability to activate macrophages, leads to improvements by reducing the injured area and not by improving the ability of neurons to regenerate (Benowitz and Popovich, 2011).

Injured CNS axons usually fail to form a growth cone and instead originate a non-regenerative structure called retraction bulb. The formation of a growth cone instead of a retraction bulb is absolutely essential to start regeneration (Bradke et al., 2012). Recent studies have focused on promoting regeneration by the direct action on the injured axon tip, increasing the possibility of growth cone formation. Cytoskeleton components are the strongest candidates to promote the formation of a growth cone. Indeed, the use of taxol stabilizes microtubules and promotes the formation of growth cones which can overcome the myelin

inhibitory effect (Erturk et al., 2007). The use of taxol was also shown to improve axonal regeneration following SCI (Hellal et al., 2011).

Most of the therapies referred so far require local administration at the injury site, either by local injection of cells, or by the implantation of osmotic pumps to deliver antibodies or drugs. This type of treatment, involves at least one invasive procedure, increasing the risk of complications in patient's recovery. As such, the use of electrical stimulation in a non-invasive manner for treatment poses an attractive alternative. However, the effects of electrical stimulation are not consensual, although it has been reported as beneficial following SCI being able to promote regeneration of dorsal column fibers by increasing their intrinsic ability to regenerate (Udina et al., 2008).

7.3. Combined therapies

Although many single therapies are able to improve recovery following SCI in animal models, the effects produced are usually minimal. The use of multiple approaches might be able to combine all the small improvements and in many instances have a synergistic effect on regeneration. Usually combinatorial therapies are constituted at least by one method to improve the intrinsic regeneration ability of neurons and a therapy to reduce the inhibitory effects within the injury site.

The use of tissue or cell transplantation for bridging, together with phosphodiesterase inhibitor to increase the levels of cAMP promote recovery to levels higher than any individual therapy (Nikulina et al., 2004; Pearse et al., 2004). MSC transplantation together with NT-3 treatment was also reported to promote robust regeneration within the glial scar (Lu et al., 2007). Other methods to decrease the inhibitory effect of the glial scar like the use of chondroitinase ABC together with other therapies like zymosan or neurotrophins act synergistically to promote regeneration (Tropea et al., 2003; Steinmetz et al., 2005; Garcia-Alias et al., 2011).

The use of combinatorial therapies provides a wide range of possibilities without a limit to the number of therapies that can be used at the same time. Two recent studies show cumulative benefits of using four different therapies:

increase the intrinsic growth capacity of axons by dcAMP delivery or conditioning, MSC transplantation together with NT3 delivery and the creation of a chemoattractive gradient of NT-3 across the injury site (Lu et al., 2004; Kadoya et al., 2009) (Fig. 23).

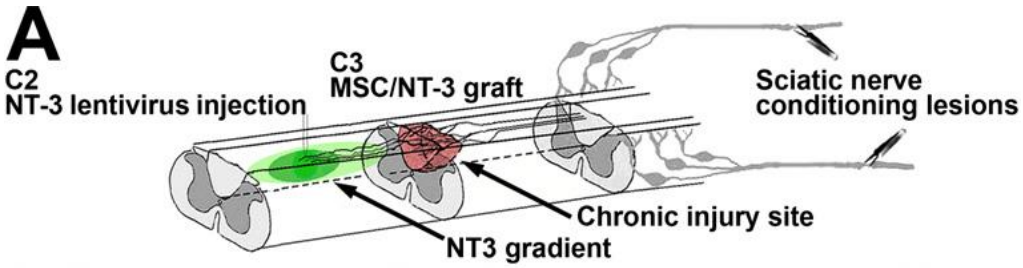

Figure 23. Combined approach to treat SCI. Example of a combined approach using lentivirus expressing NT-3 rostral to the injury, together with MSC and NT-3 at the injury site to form bridges and conditioning injury to increase the intrinsic growth ability of DRG neurons. Adapted from (Kadoya et al., 2009).

In the last two decades, the axonal regeneration field had important developments that allowed us to understand novel mechanisms on how the environment interferes in axonal regrowth and on how the intrinsic ability of neurons to regenerate is controlled. Despite all these improvements, the study of axonal regeneration of CNS neurons still did not lead to the development of any effective clinical treatment. As such, further knowledge is needed to improve either axonal regeneration or sprouting to an extension that may have meaning on the human scale.

Review

EMBO reports

Cell intrinsic control of axon regeneration

Fernando M Mar[1], Azad Bonni[2,3] & Mónica M Sousa[1,*]

Abstract

Although neurons execute a cell intrinsic program of axonal growth during development, following the establishment of connections, the developmental growth capacity declines. Besides environmental challenges, this switch largely accounts for the failure of adult central nervous system (CNS) axons to regenerate. Here, we discuss the cell intrinsic control of axon regeneration, including not only the regulation of transcriptional and epigenetic mechanisms, but also the modulation of local protein translation, retrograde and anterograde axonal transport, and microtubule dynamics. We further explore the causes underlying the failure of CNS neurons to mount a vigorous regenerative response, and the paradigms demonstrating the activation of cell intrinsic axon growth programs. Finally, we present potential mechanisms to support axon regeneration, as these may represent future therapeutic approaches to promote recovery following CNS injury and disease.

Keywords axon regeneration; axonal transport; conditioning lesion; microtubule dynamics

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See the Glossary for abbreviations used in this article.

Introduction

During development, by implementing a transcription-dependent program relying on multiple signaling pathways, neurons display robust elongation capacity that allows them to reach their targets. Following this initial phase, with the establishment of connections the developmental axon elongation ability declines. The view that neurons in the adult CNS permanently lose their intrinsic ability to grow axons was challenged by seminal studies by the Albert Aguayo group, showing that the use of peripheral nerve "bridges" in the spinal cord permits CNS axons to grow for considerable distances following injury [1]. These studies demonstrated that adult CNS neurons can activate a cell intrinsic program that supports axonal regrowth, provided that a permissive environment is created. These initial reports fueled efforts to characterize the extrinsic cues that inhibit axon growth in the CNS, while the cell intrinsic mechanisms that govern axon regeneration remained poorly understood. Several decades later, the body of knowledge gathered supports the view that counteracting or removing the extracellular inhibitory molecules results in incomplete axon regeneration *in vivo* and that a better understanding of cell intrinsic mechanisms regulating axon growth following injury is needed [2].

In contrast to the CNS, adult peripheral nervous system (PNS) axons can spontaneously regrow to a significant extent and are often used as a model to identify the players that promote axon regeneration. The regenerative capacity of the PNS is supported by the combination of extrinsic and intrinsic factors that generate a growth-permissive milieu where the execution of a cell intrinsic program leads to successful axonal regrowth. Cell intrinsic changes induced by a PNS injury can be observed *in vitro* and *in vivo,* as will be discussed in the context of the conditioning lesion paradigm. In CNS neurons, the combined action of repressors of axonal growth, the limited injury signaling mechanisms, and the lack of robust expression of regeneration-associated genes (RAGs) results in a restricted potential to regenerate. Here, we will provide a critical perspective of our understanding of the intrinsic mechanisms controlling axonal regeneration in the adult nervous system. With the term cell intrinsic, we refer to mechanisms that do not strictly depend on external cues, although external cues can influence their activity. As such, this review is not restricted to the discussion of changes in the expression of the neuronal genetic program, that is, transcriptional and epigenetic mechanisms and regulation of translation, but is extended to the analyses of intracellular pathways and mechanisms—including axonal transport and microtubule dynamics—that regulate axon growth and regeneration.

Cell intrinsic mechanisms of axonal regeneration in the PNS

Calcium influx into the axoplasm is one of the first signals caused by injury, and the depolarization triggered by the inversion of the calcium/sodium flux travels along the axon to the cell body. Calcium influx is here discussed in the context of the cell intrinsic factors that govern axon regeneration as it elicits various cell autonomous mechanisms necessary for successful axon growth, ranging from the regulation of intracellular pathways to the generation of

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Glossary

AKT	protein kinase B
APC	anaphase-promoting complex
Arg1	arginase 1
ATF3	activating transcription factor 3
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CREB	cAMP response element-binding protein
DLK	dual leucine zipper kinase
DRG	dorsal root ganglia
EFA-6	exchange factor for Arf6
Elk-1	ETS domain-containing protein
ERK	extracellular signal-regulated kinase
GAP-43	growth-associated protein-43
GSK3	glycogen synthase kinase 3
HDAC	histone deacetylase
IL-6	interleukin-6
JAK	janus kinase
JNK	c-Jun amino-terminal kinase
KIF3C	kinesin family member 3C
KLP-7	kinesin-like protein 7
MAP1B	microtubule-associated protein 1B
MAPK	mitogen-activated protein kinase
mTOR	mammalian target of rapamycin
NLS	nuclear localization signal
NPY	neuropeptide Y
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PNS	peripheral nervous system
PTEN	phosphatase and tensin homolog
RAG	regeneration-associated gene
RanBP1	Ras-related nuclear protein binding protein 1
RGC	retinal ganglion cell
SC	slow component of axonal transport
SOCS3	suppressor of cytokine signaling 3
Sox11	SRY-related HMG-box 11
STAT3	signal transducer and activator of transcription 3
TSC2	tuberous sclerosis complex 2
UTR	untranslated region
VIP	vasointestinal peptide
ZBP1	zipcode-binding protein 1

epigenetic changes. In *Caenorhabditis elegans* sensory neurons, the amplitude of the axonal calcium waves correlates with the extent of regeneration, and conversely, inhibition of voltage-gated calcium channels, or of calcium release from internal stores, reduces the regenerative growth of axons [3]. Although the consequences of electrical stimulation produce conflicting results, possibly due to differences in stimulation paradigms, a weak stimulus may improve the regeneration of rat motor [4] and sensory neurons [5]. However, a strong electrical pulse mimicking the physiological activity of adult rodent dorsal root ganglia (DRG) neurons strongly inhibits axon outgrowth, and loss of electrical activity following PNS injury promotes axonal regeneration in the PNS [6].

Independently of the electrical activity generated by calcium influx, the calcium transient activates intracellular signaling required for resealing the axonal membrane in giant squid axons [7], for local protein synthesis after optic nerve crush in rats [8,9] and for the assembly of a competent growth cone after axotomy of Aplysia buccal neurons [8,9]. Besides, calcium influx activates calcium-dependent enzymes including adenylate cyclase, leading to increased cAMP levels that signal to the downstream dual leucine zipper kinase (DLK-1) promoting the local transformation of the cytoskeleton needed for growth cone assembly in *C. elegans* sensory neurons [3] (Fig 1). In mouse sensory neurons, the calcium wave requires calcium release from internal stores in addition to voltage-gated calcium channels [10]. Importantly, this back-propagating calcium wave invades the soma causing protein kinase Cμ (PKCμ) activation followed by nuclear export of histone deacetylase 5 (HDAC5), thereby increasing histone acetylation and activating the proregenerative transcription program [10] (Fig 1). This epigenetic mechanism controls the switch from non-elongating to growth-competent axons [10]. This early and fast calcium-dependent phase of injury signaling has been suggested to prime the neuronal cell body to the signals that will be conveyed later, after microtubule-dependent retrograde transport along the axon [10].

The importance of increasing histone acetylation to induce axonal regeneration has also been demonstrated using the HDAC inhibitor valproic acid, which improves the outcome in a rat model of spinal cord injury [11]. Further reinforcing the link between increased axon growth and histone acetylation, in mouse DRG neurons triggered into a growth state, as is the case following conditioning lesion (this model is discussed below), histone 4 acetylation is restored and RAG transcription is initiated [12]. During this epigenetic reprogramming, histone-modifying enzymes together with Smad1 facilitate the transcriptional activation of RAGs, including neuropeptide Y (NPY), vasointestinal peptide (VIP), Sprr1a, and galanin, thus providing a link between transcription factors and RAGs through epigenetic regulation [12]. Importantly, the Smad1 pathway has been recently shown to be central for promoting rat sensory axon regeneration [13]. Several other epigenetic mechanisms have been reported in the context of axon regrowth [14]. The histone acetyltransferases CBP/p300 acetylate histone 3 at K9-14 and the transcription factor p53, thereby initiating a proregenerative transcriptional program that regulates RAG expression in rodents [15–17]. In this context, p300 directly occupies and acetylates histones in the promoters of the growth-associated protein 43 (GAP-43), coronin 1 b, and Sprr1a driving the expression of several RAGs [17]. The importance of this mechanism is highlighted by the observation that, in the model of optic nerve crush, overexpression of p300 promotes axonal growth [17]. Besides, axonal regeneration in the rodent CNS after spinal cord injury is dependent on the folate pathway through DNA methylation [18]. A thorough comparison of the epigenetic landscape in regenerative and non-regenerative conditions is required to translate the knowledge gathered in this field into novel therapeutic approaches [19].

Retrograde transport of locally activated injury signals

In addition to calcium-mediated signaling, studies using Aplysia neurons described the first injury signals capable of communicating lesion from the injury site to the cell body [20,21]. In Aplysia, injection of axoplasm from injured nerves into naïve neurons triggers an injury-like behavior accompanied by increased growth. The model proposed to explain this behavior has been that phosphorylation of injury signals exposes hidden nuclear localization signals (NLS),

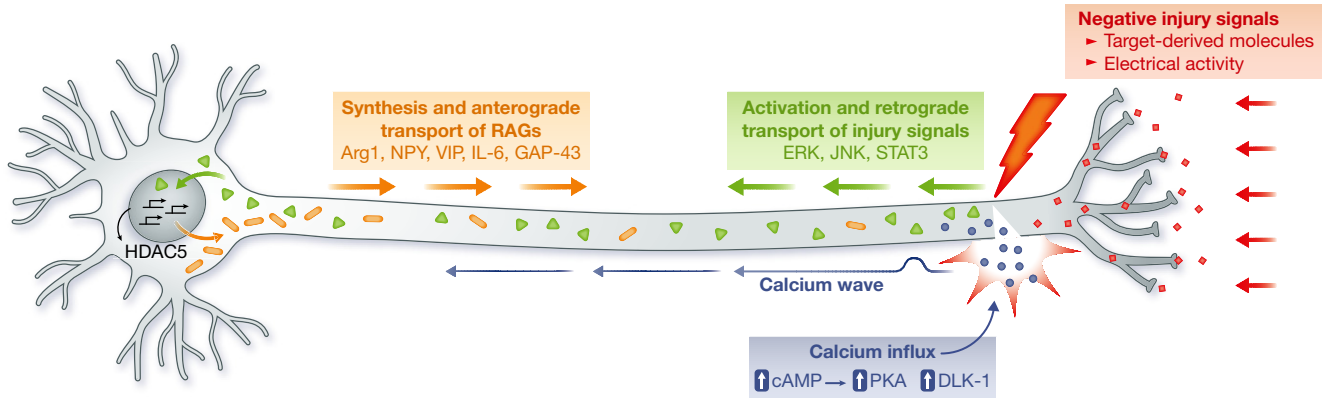

Figure 1. The injury response of a PNS neuron.
Repression of axonal elongation can be relieved upon injury through the interruption of target-derived negative injury signals and electrical activity. Calcium influx into the axoplasm activates cAMP and PKA, signaling to DLK-1 and promoting growth cone formation, local protein synthesis, and resealing of the axonal membrane. The calcium wave back-propagates to the cell body, leading to HDAC5 nuclear export, activating the proregenerative transcription program. Following the calcium-dependent early phase, retrograde transport of injury signals including ERK, JNK, and STAT3 occurs. In the cell body, RAGs (Arg1, NPY, VIP, IL-6, GAP-43, among others) that are necessary to mount a regenerative response are expressed. cAMP, cyclic adenosine monophosphate; DLK-1, dual leucine zipper kinase 1; ERK, extracellular signal-regulated kinase; HDAC5, histone deacetylase 5; JNK, c-Jun amino-terminal kinase; RAG, regeneration-associated gene.

targeting them to the nucleus [22]. The importance of retrograde transport of NLS-containing proteins has been demonstrated later in rats, as injection of an NLS synthetic peptide into the injured nerve competes with the activation of intrinsic growth programs by preventing the retrograde transport of injury signals [23]. NLS-containing proteins bind with low affinity to importin-α, the only importin present in intact nerves, but with high affinity to importin-α/β heterodimers. Following injury, local translation of importin-β at the injury site leads to the formation of importin-α/β heterodimers, which bind to NLS-containing proteins and are retrogradely transported to the cell body [23]. In fact, axonal localization of importin-β mRNA is essential for the correct assembly of the retrograde transport machinery of injury signals as demonstrated in rodent DRG neurons [24]. In the case of ERK, its binding to the retrograde transport machinery is not dependent on an NLS signal. Instead, ERK is linked to the retrograde transport machinery through locally synthesized vimentin [25] (Fig 2), as further discussed below. Besides importin-β, Ras-related nuclear protein binding protein 1 (RanBP1) is also synthesized locally after injury, allowing the binding of importin-α/β heterodimers to dynein in rat DRG neurons [26]. Below, the importance of local protein translation in axon regeneration will be further discussed.

Several injury signals locally activated and retrogradely transported to the cell body have been identified using mostly rat or mouse DRG neurons and sciatic nerve injury as a model: extracellular signal-regulated kinase (ERK) [25], c-Jun N-terminal kinases (JNK) [27], and signal transducer and activator of transcription 3 (STAT3) [28] (Fig 1). In the case of ERK, the use of MEK1,2 inhibitors following peripheral nerve injury reduces the regenerative response, suggesting that MEK may phosphorylate ERK at the injury site [25]. To overcome the challenge of transporting phosphorylated signals from the injury site to the cell body, protection mechanisms

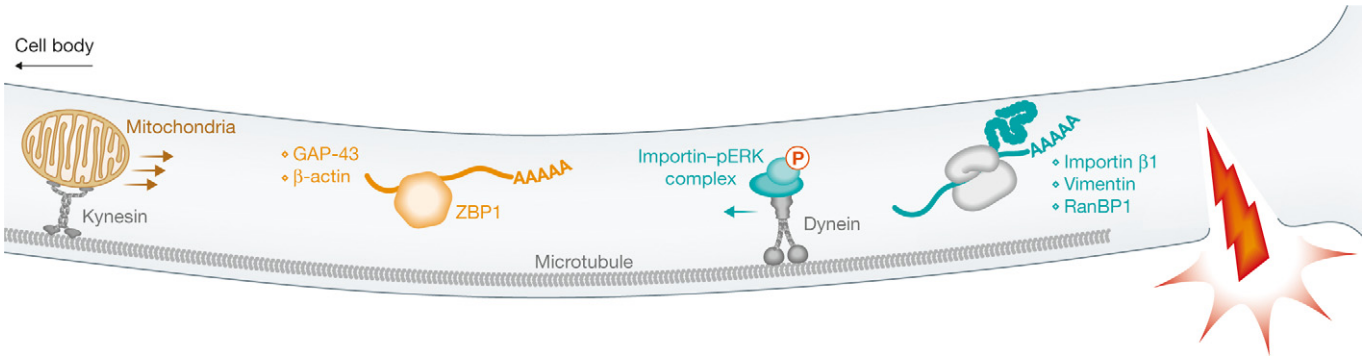

Figure 2. Injury induces profound changes in axonal transport and local protein synthesis.
Following injury, local protein synthesis is activated. Axotomy triggers the translation of importin β1 and vimentin mRNAs. Vimentin links pERK to the importin–dynein complex such that the injury signal is retrogradely transported to the cell body. ZBP1 is required for the axonal localization of β-actin and GAP-43 mRNAs that are translated after injury. Increased anterograde transport of mitochondria is also elicited by injury. GAP-43, growth-associated protein-43; pERK, phosphorylated extracellular signal-regulated kinase; ZBP1, zipcode-binding protein 1.

are in place. As discussed above, vimentin, for example, binds to phosphorylated ERK, which enables not only linkage to the retrograde transport machinery but also hinders ERK dephosphorylation [25] (Fig 2). Although pERK is involved in the retrograde signal that initiates regeneration, it is probably not required during subsequent outgrowth [25]. Also, the formation of the JNK-Sunday Driver complex allows the signal to be transported on vesicular structures linked to the transport machinery, possibly protecting it from dephosphorylation [29]. Importantly, JNK signaling has been implicated in the reorganization of the axonal cytoskeleton and in neurite regeneration [30]. In the case of STAT3, it is interesting to note that besides contributing to axonal regeneration [31], its activation is also important for neuronal survival after injury [28].

The response to nerve injury relies on the activation of numerous transcription factors. Some of the transcription factors are activated by the above injury signals. ERK activates ETS domain-containing protein (Elk-1) [26], while JNK activates c-Jun and activating transcription factor 3 (ATF3) [32]. Other transcription factors involved in the regenerative response have also been identified in assays using rat and mouse DRG neurons, including cAMP response element-binding protein (CREB) [31], SRY-related HMG-box (Sox11) [33], phosphatidylinositol 3-kinase (PI3K), and Smad1 [13,34]. Together, they alter the transcriptional profile of injured neurons contributing to their survival and regeneration [33]. In this context, and integrating previous data, it has been demonstrated that PNS injury activates PI3K signaling, leading to the inactivation of glycogen synthase kinase 3 (GSK3) and suggesting that the PI3K-GSK3-Smad1 pathway is central for promoting sensory axon regeneration [13]. The activated transcription factors also induce the expression of several RAGs including arginase-1 [34], NPY, VIP [35], interleukin-6 (IL-6) [36], GAP-43, and CAP-23 [37], among others (Fig 1).

As a result of injury, the interruption of retrograde transport of negative injury signals, possibly target-derived molecules, might release neurons from the repression to elongate, allowing regeneration to take place. In this context, it has been demonstrated that following lesion, reduction in nerve growth factor (NGF) levels in sympathetic and sensory neurons contributes to the increased levels of neuropeptide expression [38]. Likewise, cessation of electrical activity after peripheral lesion contributes to the regenerative response [6]. As such, both target-derived NGF and electrical activity are seen as negative injury signals. In summary, in addition to the presence of regeneration-promoting injury signals, in adult naïve PNS neurons repression of axonal elongation might be relieved upon injury.

Anterograde axonal transport for an effective regenerative response

As neurons are highly polarized cells, proteins synthesized as a response to injury signaling need to travel from the cell body to the distant axon tip. Thus, the control of anterograde axonal transport is an intracellular mechanism of pivotal importance for axon regeneration. Anterograde axonal transport is divided into the slow component a (SCa) that transports neurofilaments, tubulin, and microtubule-associated proteins; the SCb that transports cytoplasmic proteins, such as glycolytic enzymes and actin; and the fast component that transports vesicles and membranous organelles [39]. Surprisingly, the motors of both slow and fast components are similar, and the different average rates are due to the pausing behavior of cargoes during transport [40]. The flux of anterograde axonal transport elicited by injury needs to supply the axon with structural components (tubulin, actin, and neurofilaments), synaptic and cytosolic proteins, vesicles, and organelles. Interestingly, the speed of axonal regeneration is similar to the one of SCb, supporting the relevance of anterograde transport in sustaining regrowing axons [41]. Of note, following sciatic nerve injury in mice, anterograde transport of mitochondria in the proximal nerve increases by more than 80% and declines only slightly subsequently [42], which may support the increased metabolic demand of regenerating peripheral axons (Fig 2). Whether the transport of other organelles or cytoplasmic proteins is also increased remains to be clarified.

Despite the discussed evidence suggesting that axonal transport plays a central role during axonal regeneration, the modulation of transport by injury is not well understood. Specifically, the mechanisms that underlie the increase in axonal transport after PNS injury remain to be established, and future studies should determine whether molecular motors are affected by lesion or, if alternatively, microtubule tracks are modified.

Zipcodes and local protein synthesis during axonal regeneration

The relevance of local protein synthesis in axons remained obscure until recently. To date, as a consequence of several studies performed mainly in rodents, it is widely accepted that the first building blocks of regenerating axons are obtained by local protein synthesis along the axon and in the growth cone. In the adult PNS, axons contain ribosomes distributed unevenly along the axoplasm [43], and Schwann cells may also provide axonal ribosomes following injury [44]. In contrast, in the CNS, axons synthesize proteins during development in the growth cone, but polysomes are restricted to the axon initial segment in adult rodent axons [45]. Besides the correlation between the different capacities of PNS and CNS axons to locally synthesize proteins and regenerate, local protein synthesis generally decreases with axonal aging, which again coincides with a reduced regeneration potential [46]. Further supporting a critical role for local protein synthesis during axonal regeneration, application of inhibitors of protein synthesis to cut rat axons, including axons whose cell bodies were removed, decreases the number of transected axons that reform a growth cone [45]. In fact, growth cone formation after axotomy depends on local protein synthesis and degradation, controlled by the mammalian target of rapamycin (mTOR), $p38^{MAPK}$, and caspase-3-dependent pathways [45].

The identification of axonally localized mRNAs has been facilitated considerably with the development of more sensitive techniques. Genome-wide microarray analyses revealed that several mRNAs are localized axonally in rat sensory axons and that this repertoire changes substantially from development to adulthood [47,48]. Axonal mRNAs need to be actively transported, stored, and protected from degradation at their final destination. The β-actin zipcode, a conserved sequence present at the beginning of its 3′ UTR, is the sequence for mRNA axonal targeting identified in mice. [49]. This sequence interacts with the RNA-binding protein zipcode-binding protein 1 (ZBP-1), which mediates the axonal

localization of β-actin [50] (Fig 2). Reinforcing the importance of this mechanism for axonal regrowth, mice with reduced ZBP1 levels show decreased axon regeneration after sciatic nerve injury [51]. Additionally, the overexpression of β-actin's 3′ UTR was shown to compete *in vivo* with other ZBP1 cargo mRNAs such as GAP-43 [52]. It has now been demonstrated that axonal translation of β-actin supports axon branching, while that of GAP-43 promotes the elongation of rodent sensory neurons during normal axon growth [53]. These growth-promoting pathways might be relevant for regenerating axons as well. As already addressed, data supporting the local axonal translation of importin β1 have been obtained and an axon-localizing region in the 3′ UTR of importin β1 has been identified [24]. Mice lacking the axon-localizing region in the 3′ UTR of importin β1 displayed a delay in axonal regeneration of sensory neurons [24]. In summary, the above findings support the conclusion that the ability of axons to locally synthesize proteins is important for their capacity to regenerate.

Why are CNS neurons unable to mount a robust regenerative program?

In contrast to the PNS, injured CNS axons have a limited ability to regenerate. Besides the formation of a highly inhibitory glial scar, several differences can be put forward to explain this lack of regenerative capacity, including inefficient Wallerian degeneration, possible defects in injury signaling, lack of a robust response to injury, limited capacity to locally synthesize proteins, and the existence of inhibitors of axonal regrowth. Indeed, rat CNS neurons fail to effectively activate many of the genes necessary for axonal regeneration to occur [54]. Interestingly, the calcium changes in the cell body have a higher amplitude and duration in rat DRG when compared to cortical neurons [55], and DRG neurons can survive long periods of high calcium, whereas these are deleterious for CNS neurons [56]. Besides, increased histone acetylation fails to occur in retinal ganglion cells (RGCs) [10]. Together, these differences might contribute to the failure in activating a proregenerative program (Fig 3).

Significant advances have been made with the identification of intrinsic inhibitors of axon regrowth in the adult CNS in studies mainly performed by gene targeting in mice. In adult RGCs, deletion of phosphatase and tensin homolog (PTEN) promotes robust axon regeneration after optic nerve injury [57]. In the PNS, following nerve transection, adult sensory neurons depleted of PTEN also show increased axon regeneration [58]. PTEN antagonizes the action of PI3K, leading to the inactivation of protein kinase B (AKT) and of mTOR signaling. In contrast to the CNS, mTOR has been suggested to be dispensable for sensory axon regeneration [58], where instead, the PI3K-GSK3-Smad1 pathway operates [13]. However, the importance of mTOR in PNS regeneration remains to be clarified as in contrast to CNS neurons, which downregulate mTOR activity after injury, PNS neurons activate mTOR and deletion of tuberous sclerosis complex 2 (TSC2), another negative regulator of mTOR, increases sensory axon regeneration *in vivo* [59]. It is noteworthy that the observation that mTOR might be dispensable for sensory axon regeneration under physiological conditions does not necessarily contradict the result that ectopic activation of mTOR (as occurs after the deletion of TSC2) promotes axon regeneration.

Through the analysis of axon regeneration in different mutant mouse lines, deletion of the suppressor of cytokine signaling 3 (SOCS3), an inhibitor of the JAK-STAT3 pathway, has been shown to promote robust regeneration of injured optic nerve axons [60]. Of note, simultaneous deletion of PTEN and SOCS3 further increases

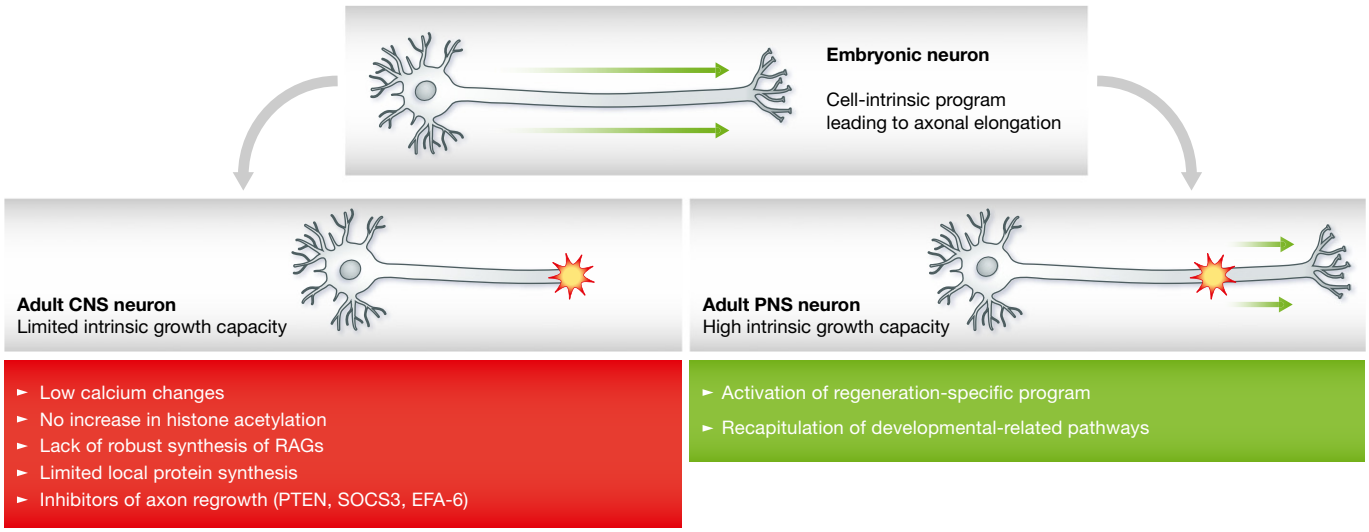

Figure 3. Increased growth capacity of PNS versus CNS neurons.
During development, through a cell intrinsic program composed of multiple pathways, neurons display a robust elongation capacity. After the establishment of connections, adult CNS neurons have a limited regenerative ability as the consequence of decreased calcium changes, no increase in histone acetylation, lack of robust synthesis of RAGs, limited local protein synthesis, and presence of inhibitors of axon regrowth (PTEN, SOCS3, and EFA-6). Adult PNS neurons have, however, a high intrinsic growth capacity as a consequence of the activation of a regeneration-specific program and probably also through the recapitulation of developmental-related pathways. CNS, central nervous system; EFA-6, exchange factor for Arf6; PNS, peripheral nervous system; PTEN, phosphatase and tensin homolog; RAG, regeneration-associated gene; SOCS3, suppressor of cytokine signaling 3.

axon regeneration, as these signals regulate two independent pathways that act synergistically [61]. In summary, the mTOR and STAT3 pathways emerged as key regulators promoting long-distance axon regeneration in the adult CNS. Reinforcing this view, PTEN or SOCS3 deletion improves regeneration of distinct CNS axons [57,60,62,63], and sustained activation of STAT3 promotes corticospinal remodeling and functional recovery after spinal cord injury [64]. Recently, in *C. elegans* mechanosensory neurons, the conserved Arf guanine nucleotide-exchange factor EFA-6 has been reported to be an intrinsic inhibitor of axon regrowth that operates by affecting axonal microtubule dynamics, acting downstream of and/or in parallel with DLK-1 [65].

Together, these studies indicate that axon regeneration is restrained not only by extrinsic inhibitory cues but also by intrinsic factors. As such, the manipulation of intrinsic growth control pathways is actively being pursued as a therapeutic approach to promote axon regeneration after CNS injury.

The cell intrinsic growth capacity of adult CNS neurons can be activated

Despite the general inability of CNS axons to regenerate, regrowth can be activated under specific conditions, for example the conditioning lesion effect. DRG neurons possess a peripheral axon branch that regenerates when injured, and a central axon branch that enters the spinal cord (forming the dorsal column fibers) and does not regenerate upon injury. However, when the peripheral branch is lesioned prior to lesioning the central branch (conditioning lesion), the central axon can overcome the glial scar inhibitory effect, regenerating to a significant extent both *in vivo* and *in vitro* [66,67], when plated on inhibitory substrates, including myelin [68]. The conditioning effect is probably due to the activation of the regenerative machinery prior to CNS lesion. Studies performed in rodents have shown that the increase in regeneration capacity encompasses RAG expression [33,69] and possibly changes in axonal transport [70]. In fact, the response to injury starts as soon as 1 day following lesion [68] and has a long-lasting effect, as RAGs are still expressed 2 months following the priming injury [71]. Although a peripheral lesion performed subsequently to the CNS injury does not improve axonal regeneration due to the assembly of a thick glial scar, it still increases the intrinsic regenerative ability of DRG neurons [71].

The conditioning effect certainly represents a good model to identify the mechanisms underlying the cell intrinsic regenerative capacity. Numerous molecular pathways have been shown to be regulated by a conditioning lesion, in accordance with the robust and broad transcriptional change that conditioning causes in DRG neurons. The initial unifying concept was that the conditioning effect was mediated by increased cAMP levels induced by injury [68]. cAMP prompted neurons to overcome myelin inhibition *in vitro*, and treatment with dibutyryl-cAMP, a cell-permeable analog of cAMP, increased the regeneration of dorsal column fibers following spinal cord injury [68,72]. Further supporting the pivotal role of cAMP, the phosphodiesterase inhibitor rolipram increases cAMP levels, leading to enhanced regeneration of serotonergic axons and functional recovery following spinal cord injury in rats [73]. However, the robust effect of cAMP in axon regeneration has been questioned by recent studies, as the use of cAMP analogs has failed to reproduce the full effect of a conditioning lesion [74]. Increased levels of cAMP appear to promote axon regeneration by overcoming myelin-based inhibitors rather than by modulating the intrinsic ability of neurons to support axon regeneration. It has been shown that elevation of cAMP fails to increase the SCb of axonal transport, the rate-limiting step of axon growth [75].

Besides cAMP, several studies have identified broad changes in gene expression in rodent conditioned neurons [36,69], regulated by the activation of multiple transcription factors [76]. Downstream, the expression of traditional RAGs such as GAP-43 and CAP-23 [77] is induced and novel RAGs have been identified using this model, including arginase-1 [34] and IL-6 [36]. However, none of the identified transcription factors or RAGs reproduces the entire conditioning effect [74], suggesting that conditioning cannot be mimicked by manipulating a single pathway. Supporting this view, epigenetic changes elicited by HDAC5 nuclear export partially reproduce the conditioning lesion effect [10]. Several questions remain open, including the differences in injury-induced signaling that allow a peripheral injury to elicit a strong regeneration response, whereas a central lesion to the same neuron fails to do so (Sidebar A).

Manipulating axonal microtubule dynamics to promote regeneration

Among the multiple processes involved in the generation of a new growth cone, cytoskeleton reorganization is crucial for the intrinsic ability to regenerate. While upon injury CNS axons form a retraction bulb with a disorganized network of microtubules, PNS axons form a growth cone with stable microtubules in the backbone and dynamic microtubules in the tip [78]. Pharmacological destabilization of microtubules converts a growth cone into a retraction bulb, and taxol-induced stabilization generates growth cones that can overcome myelin inhibition [78] and regenerate following spinal cord injury [79]. Also, HDAC6 inhibition in rodents results in increased levels of acetylated/stable microtubules and enhances the growth of sensory neurons on myelin [80]. It has been recently suggested that instead of inducing the normal mode of repair, where the axon tip might behave more dynamically, taxol might promote axonal regeneration by enabling the axon tip to become more forceful [81]. In rodent sensory neurons, HDAC5 accumulates at the tip of injured axons where local tubulin deacetylation induces growth cone microtubule dynamics and axon regeneration [82]. The varying effects that microtubule stability might have on axon regeneration do not allow for a clear causal relationship between axon regeneration and microtubule stability. Moreover, at this point, the available literature does not provide sufficient detail to allow for a comparison between the effects of HDAC5, HDAC6, and taxol on tubulin dynamics in the axon shaft versus axon tip. Although the results obtained with studies using taxol and HDAC6 seem contradictory to the data reported for HDAC5 and the kinesin family member KIF3C [83], previous studies have shown that efficient developmental axon growth requires an optimal level of microtubule dynamics. Thus, destabilizing or overstabilizing microtubules could both impair axon growth.

Many of the pathways that contribute to cell intrinsic control of regeneration participate in the remodeling of the axonal cytoskeleton, specifically by modulating microtubule dynamics. As such, potential therapeutic strategies intervening at the level of microtubule-related proteins have been actively pursued. In this respect, and besides those already discussed in this review including HDAC5 and 6, several other possible targets have been identified, including GSK3β, as many of its substrates are microtubule-interacting proteins [84].

Members of the kinesin family have also been put forward as important players in regulating microtubule dynamics during axonal regeneration. KIF3C has been shown to be an injury-specific kinesin with microtubule-destabilizing function, playing a key role during axon regrowth [83]. Depletion of KIF3C in adult neurons leads to an increase in stable and looped microtubules and delays axonal regeneration after injury [83]. In addition to KIF3C, in *C. elegans* mechanosensory axons, the depolymerizing kinesin-like protein family member 7 (KLP-7) restricts microtubule growth in the steady state [85]. After axon injury, the number of growing microtubules is increased at the injury site, simultaneously with the downregulation of KLP-7, in a cascade coordinated by DLK-1 [85]. This mechanism has been proposed to allow the stable microtubule cytoskeleton of a mature neuron to be converted into the dynamically growing microtubule cytoskeleton of a regenerating axon.

Besides the importance of identifying microtubule-interacting proteins that participate in either the formation of a retraction bulb or of a growth cone, further analysis of the regulation of post-translational microtubule modifications following injury is needed, as these control microtubule dynamics and may also interfere with axonal transport, therefore impacting on axonal regrowth.

Conclusions and outlook

Recent evidence obtained by systematic genetic screening in *C. elegans* shows that besides triggering developmental programs that may be repressed in mature neurons, regenerative growth involves specific pathways that sense and specifically respond to damage [65] (Fig 3). Although axonal regeneration cannot be viewed as mere recapitulation of axonal elongation during development, some of the operating mechanisms are shared. Axon growth during development occurs in two different phases: an initial phase of growth and a later phase that takes place during pruning [86]. Axon growth during developmental remodeling appears to be mechanistically distinct from initial axon outgrowth. Interestingly, common pathways, for example mTOR signaling, operate during developmental axon regrowth and axonal regeneration [86].

The ubiquitin ligase Cdh1-anaphase-promoting complex (Cdh1-APC) pathway is also a critical cell intrinsic mechanism that regulates axon growth in the rodent developing cerebellar cortex [87]. The inhibition of Cdh1-APC in primary neurons enhances axonal growth and also overrides myelin inhibition of axon regrowth [87]. Conceivably, Cdh1-APC might therefore potentially limit axonal growth in the adult CNS. In key follow-up studies, the transcriptional regulator SnoN has been identified as a critical substrate of Cdh1-APC in neurons [88], operating in a pathway that is regulated by transforming growth factor β-Smad2 signaling [89]. Smad2 knockdown also overrides myelin inhibition of axon growth [89]. More recently, expression of a mutant SnoN resistant to degradation has been shown to enhance axonal regeneration following spinal cord injury in rats [90]. Together, these findings raise the exciting prospect that pathways operating during development might also drive axonal regeneration following injury. Therapeutic strategies aiming at the reactivation of these pathways in injured CNS neurons might be successful in enhancing our capacity to regenerate neurons in response to injury or disease.

Sidebar A. In need of answers

(i) How is axonal transport modulated by injury? Specifically, what are the mechanisms underlying the increased axonal transport after PNS injury? Are molecular motors affected or are post-translational microtubule modifications altered?
(ii) What are the differences in injury-induced signaling that allow an injury to the peripheral branch of DRG neurons to elicit a strong regeneration response, whereas lesion to the central branch fails to elicit this response?
(iii) Is there a causal relationship between axon regeneration and microtubule dynamics?
(iv) Are developmental pathways recapitulated during axonal regeneration following injury?

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Conflict of interest

The authors declare that they have no conflict of interest.

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Research goals

The main focus of this work was to dissect mechanisms controlling axonal regeneration. As such, the following specific objectives were covered:

- Most of the known inhibitory components in the CNS are myelin associated proteins. We used the shiverer mouse that has an almost complete absence of compact myelin in the CNS, to study the impact of myelin absence in CNS axonal regeneration (chapter 1).

- The ability of PNS axons to signal injury underlies their strong response to promote regeneration. Using the dorsal root injury as model of CNS lesion, I evaluated if the CNS inability to promote regeneration could be due to differential activation of injury signals (chapter 2).

- The conditioning injury paradigm was used to identify novel regeneration enhancers that could be anterogradely transported and increase axonal regeneration (chapter 3).

Prologue – Characterization of transthyretin as an axonal regeneration enhancer in the PNS

My interest in axonal regeneration came from my initial involvement in a project that characterized transthyretin (TTR) as an axonal regeneration enhancer in the peripheral nervous system. That work was published (Fleming et al., 2009) and I contributed as the second author.

In this publication that is displayed in the following pages, I performed the experiments described in Fig. 2*A*, Fig. 6*C* and Fig. 8*C*. I have also performed the retrograde labeling of DRG neurons with cholera toxin B (page 3226).

In summary, the experiments that I conducted were pivotal to show that:

- TTR can be delivered to the sciatic nerve following injury.
- TTR KO mice have decreased axonal retrograde transport.
- TTR internalization by DRG neurons is megalin-dependent
- TTR endocytosis by DRG neurons is clathrin-mediated

Development/Plasticity/Repair

Transthyretin Internalization by Sensory Neurons Is Megalin Mediated and Necessary for Its Neuritogenic Activity

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Mutated transthyretin (TTR) causes familial amyloid polyneuropathy, a neurodegenerative disorder characterized by TTR deposition in the peripheral nervous system (PNS). The origin/reason for TTR deposition in the nerve is unknown. Here we demonstrate that both endogenous mouse TTR and TTR injected intravenously have access to the mouse sciatic nerve. We previously determined that in the absence of TTR, both neurite outgrowth *in vitro* and nerve regeneration *in vivo* were impaired. Reinforcing this finding, we now show that local TTR delivery to the crushed sciatic nerve rescues the regeneration phenotype of TTR knock-out (KO) mice. As the absence of TTR was unrelated to neuronal survival, we further evaluated the Schwann cell and inflammatory response to injury, as well as axonal retrograde transport, in the presence/absence of TTR. Only retrograde transport was impaired in TTR KO mice which, in addition to the neurite outgrowth impairment, might account for the decreased regeneration in this strain. Moreover, we show that *in vitro*, in dorsal root ganglia neurons, clathrin-dependent megalin-mediated TTR internalization is needed for TTR neuritogenic activity. Supporting this observation, we demonstrate that *in vivo*, decreased levels of megalin lead to decreased nerve regeneration and that megalin's action as a regeneration enhancer is dependent on TTR. In conclusion, our work unravels the mechanism of TTR action during nerve regeneration. Additionally, TTR presence in the nerve, as is here shown, may underlie its preferential deposition in the PNS of familial amyloid polyneuropathy patients.

Introduction

When mutated, transthyretin (TTR) is related to familial amyloid polyneuropathy (FAP) (Saraiva 2001), a neurodegenerative disorder characterized by extracellular deposition of TTR aggregates and amyloid fibrils, particularly in the peripheral nervous system (PNS) (Andrade, 1952). As a consequence of TTR deposition, axonal degeneration arises, ending up in neuronal loss. Several clues have emerged as to the molecular mechanisms of TTR-mediated cellular toxicity leading to neurodegeneration in FAP (Sousa and Saraiva, 2003). The origin of TTR deposited in the PNS of FAP patients is however unknown. TTR is mainly synthesized by the liver and the choroid plexus that are respectively the sources of TTR in the plasma and CSF. TTR was reported as being expressed in the PNS, namely by glial cells of dorsal root ganglia (DRG) (Murakami et al., 2008). However, this issue was clarified in a subsequent study showing that TTR synthesis does not occur in DRG cells (Sousa and Saraiva, 2008). Under physiological conditions serum-free human and rat nerve endoneurial fluid display TTR immunoreaction (Saraiva et al., 1988). TTR may have access to the nerve through the blood-nerve barrier (BNB), and/or through contact between peripheral nerve roots and CSF, where TTR is present in high levels. In fact, in recipients of FAP livers, i.e., after domino liver transplantation, TTR deposits are found within the nerve of the recipients, suggesting that plasma TTR (synthesized in the liver) can cross the BNB (M. M. Sousa et al., 2004).

Apart from being a transporter of thyroxine (T_4) and retinol, TTR has been described as having functions related to the nervous system, namely to be involved in cognition (Brouillette and Quirion, 2008), behavior (J. C. Sousa et al., 2004), and neuropeptide processing (Nunes et al., 2006). In the case of the PNS, it was demonstrated that TTR enhances peripheral nerve regeneration (Fleming et al., 2007). In that study, TTR knock-out (KO) mice presented delayed functional and morphological recovery after nerve injury, as assessed from a decreased number of myelinated and unmyelinated axons in the course of regeneration. TTR capacity to enhance nerve regeneration was unrelated to neuronal survival as the absence of TTR was not accompanied by increased neuronal loss. In transgenic mice expressing human TTR in neurons, in a TTR KO background, the delayed regeneration of TTR KO mice was rescued, reinforcing that TTR enhances nerve regeneration (Fleming et al., 2007). Additionally, absence of TTR was found to be related to a decreased ability of DRG neurons to

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grow neurites *in vitro*: cells grown without TTR displayed a reduced number of neurites, as well as a decreased size of the longest neurite (Fleming et al., 2007). This TTR neuritogenic effect was demonstrated to be independent of its major ligands, retinol and T_4. The newly described TTR role in peripheral nerve biology might explain why, when mutated, the protein preferentially accumulates in the PNS. Yet, the means through which TTR increases regeneration is unknown.

In the current work, we aimed at finding the mechanism through which TTR enhances nerve regeneration and neurite outgrowth.

Materials and Methods

Mice. Mice were handled according to European Union and National rules. WT and TTR KO (Episkopou et al., 1993) littermates (in the 129/Sv background), as well as megalin heterozygous [MEG (+/−), kindly provided by Dr. Thomas Willnow, Max-Delbrueck Center for Molecular Medicine, Berlin, Germany] and TTR KO/MEG (+/−) littermate mice (in the 129/Sv background), were obtained from the offspring of heterozygous breeding pairs. All animals were maintained under a 12 h light/dark cycle and fed with regular rodent's chow and tap water *ad libitum*. Genotypes were determined from tail extracted genomic DNA. Unless otherwise stated, all comparisons comprised groups of 6 animals per genotype, age- and sex-matched. For TTR detection in the nerve and nerve injury experiments, either 3 or 6 months old animals were used. For DRG neuron cultures, animals with 1–4 weeks of age were used. All experiments were performed with the observer blinded to the animal's genotype.

Nerve injury. Mice were anesthetized with medetomidine/ketamine and a 4-mm-long incision was made in the shaved thigh skin. For nerve crush, the sciatic nerve was exposed and crush was performed using Pean forceps, twice during 15 s. To standardize the procedure, the crush site was maintained constant for each animal at 35 mm from the tip of the third digit. A single skin suture, immediately above the crush site, served as an additional reference. After surgery, animals were allowed to recover for 5, 15, 30, or 75 d. For chronic constriction injury (CCI), the sciatic nerve was exposed and one ligature was tied around the nerve using 4/0 silk suture material (Braun). Skin was sutured and mice were allowed to recover for 24 h. Mice were perfused for 20 min with PBS through the vena cava at a flow rate of 2 ml/min and the sciatic nerve was subsequently collected.

Assessment of mouse TTR presence in the nerve after nerve crush. WT mice underwent nerve crush and were allowed to recover for 3 d. Mice were perfused for 20 min with PBS through the vena cava at a flow rate of 2 ml/min and the distal nerve segments were subsequently collected. Western blot was performed as described below. The primary antibody used was a custom made rabbit anti-mouse TTR antibody (produced against recombinant mouse TTR, 1:500). Electronic microscopy using WT distal nerve segments was performed as described below.

Assessment of TTR access to the nerve. TTR was produced as previously described (Liz et al., 2004), and 1 mg of recombinant human TTR was conjugated with Alexa 488 using the Alexa Fluor 488 labeling kit (Invitrogen), according to the manufacturer's instructions. hTTR-Alexa 488 was separated from free Alexa 488 by fine size exclusion chromatography in Bio-Rad BioGel P-30 resin columns. Subsequently, 1 μg of hTTR-Alexa 488 was run in a 15% SDS polyacrylamide gel and, after electrophoresis, hTTR-Alexa 488 was visualized in a Typhoon 8600 (Amersham) to check labeling efficacy. hTTR-Alexa 488 (100 μg) was injected intravenously in the tail vein of WT and TTR KO mice. The next day, mice were subjected to unilateral nerve crush as described above. The following day, mice were killed, and both crushed and contralateral sciatic nerves were collected and cryoprotected with a 1.2% L-lysine solution containing 2% formalin. For immunohistochemistry using the primary rabbit anti-human TTR (Dako; 1:1500), and rabbit anti-mouse TTR (1:2000) polyclonal antibodies, 10 μm-thick sections were incubated in 0.1% sodium borohydride (Sigma) for 5 min. Sections were then blocked in blocking buffer (1% bovine serum albumin and 4% fetal bovine serum in PBS) for 1 h at room temperature, and incubated with primary antibody diluted in blocking buffer overnight. As a negative control, slides were left overnight at 4°C with either anti-mouse (TTR previously adsorbed with recombinant mouse TTR, produced as previously described (Liz et al., 2004), or with blocking buffer alone. The adsorption of anti-mouse TTR was performed by incubation of 200 μg of recombinant mouse TTR with 1 μl of anti-mouse TTR overnight at room temperature with agitation. After centrifugation at 16,000 g, the supernatant, diluted 1:2000 in blocking buffer, was used to perform immunohistochemistry. Subsequently, slide incubation with the anti-rabbit IgG-Alexa 568 secondary antibody (Invitrogen, 1:1000) diluted in blocking buffer was performed for 1 h at room temperature. Coverslips were mounted with VectaShield Mounting Medium with DAPI (Vector), and images were taken using a Leica SP2 AOBS SE (Leica) confocal laser scanning microscope.

Local delivery of TTR to the crushed nerve. WT and TTR KO mice underwent bilateral nerve crush as described above. Immediately after crush and before suturing, 80 μl of Matrigel Basement Membrane Matrix (BD Biosciences) was applied to the left sciatic nerve crush site; the right sciatic nerve crush site received 80 μl of Matrigel Basement Membrane Matrix supplemented with 60 μg of recombinant WT TTR, produced as previously described (Liz et al., 2004). Ten minutes after Matrigel application, when it had already gelled, skin was sutured. The animals were allowed to recover for either 15 or 30 d. Mice were killed using a lethal anesthesia dosage, and the left and right nerve distal stumps were collected and processed for morphometric analysis as described below. To assess for the presence of TTR in the nerve after Matrigel application, similar experiments were performed where 80 μl of Matrigel supplemented with 60 μg of hTTR-Alexa 488 (produced as described above) were applied to the right sciatic nerve crush site of TTR KO mice; to the left sciatic nerve crush site, Matrigel supplemented with free Alexa 488 (equivalent to the amount of fluorophore present in 60 μg of hTTR-Alexa 488) was applied. As an additional negative control, a group of animals received Matrigel alone in the sciatic nerve crush site. Mice were killed 1 d after Matrigel application and the nerve (3–4 mm upstream and downstream of the crush site) was collected, cryoprotected with a 1.2% L-lysine solution containing 2% formalin and sectioned at 8 μm. The presence of hTTR-Alexa 488 was detected using a using a Leica SP2 AOBS SE (Leica Microsystems) confocal laser scanning microscope.

Morphometric analysis. The 3 mm segments immediately distal to the crush site were fixed overnight in 1.25% glutaraldehyde in 0.1 M sodium cacodylate, washed in 0.1 M sodium cacodylate for 30 min, postfixed in 1% osmium tetroxide in 0.2 M sodium cacodylate for 60 min, washed again in 0.1 M sodium cacodylate for 30 min, dehydrated using a series of graded alcohols and propylene oxide, and embedded in epon. Transverse sections (1.0 μm thick) were cut with a SuperNova, Reichest, Leica ultramicrotome, and stained with 1% toluidine blue in an 80°C heating plate for 20 s. For each animal, the total number of myelinated fibers present in one semithin section was determined by counting 50× magnified photographs covering the whole nerve area. To determine the density of unmyelinated fibers, ultrathin transverse sections were cut and stained with uranyl acetate and lead citrate. For each animal, 20 nonoverlapping photomicrographs (7000× amplification) corresponding to ~9000 μm^2 of each ultrathin section were taken using a transmission electron microscope (Zeiss 10C) and analyzed. To assess possible differences in nerve total areas between strains, these were determined from 10× magnified photos of sciatic nerve transverse sections.

Analysis of Schwann cell proliferation. WT and TTR KO mice underwent bilateral nerve crush and were allowed to recover for 5 d. To label dividing cells, 100 μg/g body weight of BrdU (5-bromo-2′-deoxyuridine, Sigma) was injected intraperitoneally 4 and 2 h before kill. Labeling of proliferating cells was performed 5 d after injury, since at this time point, Schwann cells reach their maximum proliferative activity in injured sciatic nerves (Cheng and Zochodne, 2002). Mice were killed using a lethal anesthesia dosage and the sciatic nerves were collected and processed for immunohistochemistry. Nerves were excised, fixed in 4% neutral buffered formalin and embedded in paraffin. Sections (5 μm thick) were deparafinated in histoclear (National Diagnostics) and hydrated in a descendent alcohol series. Antigen unmasking was done by incubation in 2N HCl for 20 min at 37°C, followed by neutralization with 0.1 M $Na_2B_4O_7$, and incubation with trypsin-EDTA (Invitrogen) for 10 min at

37°C. Immunohistochemistry using the primary anti-BrdU monoclonal antibody (1:1000, Sigma) was performed with the MOM kit (Vector) according to the manufacturer's instructions, using diaminobenzidine (Sigma) as substrate. Slides were counterstained with hematoxylin (Merck). Two longitudinal nerve sections per animal were immunostained; four 50× magnified photographs per section distal to the crush site were taken, covering an area of ~0.5 mm^2, and the number of BrdU-labeled nucleus was determined, as well as the total number of nucleus stained with hematoxilin. The percentage of BrdU-labeled nucleus was then calculated for each animal.

Assessment of apoptosis by TUNEL analysis. The comparison of apoptotic cells in crushed nerves from WT and TTR KO mice was performed with the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Millipore Bioscience Research Reagents), according to the manufacturer's instructions, using diaminobenzidine (Sigma) as substrate. Slides were counterstained with hematoxylin (Merck). Eight 10× magnified photographs per section near the crush site were taken, covering an area of ~0.15 mm^2. For each animal, the number of labeled nucleus was determined, as well as the total number of nucleus stained with hematoxilin. The percentage of apoptotic nucleus was then calculated for each animal.

Determination of macrophage number. Semithin sections stained with toluidine blue of WT and TTR KO sciatic nerve distal segments were obtained as described above for morphometric analysis. For each animal, the density of macrophages present in one semithin section covering the whole nerve area was determined by observation of 20× magnified photographs 15, 30, and 75 d after nerve crush.

Western blot. Intact and CCI nerves were sonicated in 0.5% Triton X-100 (Sigma) containing protease inhibitor mix (Amersham). Protein (12 μg total per lane) was run in 15% SDS polyacrylamide gels. After electrophoresis, samples were transferred to a nitrocellulose membrane (Amersham), blocked with blocking buffer (5% nonfat dried milk in PBS), and incubated overnight at 4°C with primary antibodies diluted in blocking buffer, namely, rabbit polyclonal anti-$p75^{NTR}$ (Santa Cruz Biotechnology, 1:200), and mouse monoclonal anti-β-actin (Sigma; 1:5000). Subsequently, incubation with horseradish peroxidase (HRP)-labeled secondary antibodies diluted in blocking buffer, namely either anti-rabbit IgG-HRP (The Binding Site; 1:10,000) or anti-mouse IgG-HRP (The Binding Site; 1:5000), was performed for 1 h at room temperature. Blots were developed using the ECL PlusTM Western blotting reagents (Amersham) and exposed to Hyperfilm ECL (Amersham). Quantitative analysis of Western blots was performed using the ImageQuant software (Amersham). Results are shown as the ratio between $p75^{NTR}$ and β-actin signals.

Primary cultures of DRG neurons. Primary cultures of DRG neurons were performed as described previously (Lindsay, 1988). Briefly, DRG were dissected aseptically from WT or TTR KO mice, freed of roots and treated with 0.125% collagenase (Sigma) for 3 h at 37°C. After enzyme treatment, a single-cell suspension was obtained by trituration with a fire-polished Pasteur pipette. The cell suspension was centrifuged into a 15% albumin gradient for 10 min at 200 g. The obtained pellet was resuspended in Neurobasal medium supplemented with B27, penicillin-streptomicin, glutamine, fungizone (all from Invitrogen) and 50 ng/ml NGF (Sigma), plated in poly-L-lysine coated 13 mm coverslips and maintained at 37°C.

Transferrin transport assay. The use of human transferrin conjugated with Texas red (Tf-TR, Invitrogen) as a tracer to examine retrograde transport in cultures of DRG neurons has been previously documented (Liu et al., 2003). WT and TTR KO DRG neurons were cultivated as described above. After 3 d in culture, cells were incubated with medium containing 50 μg/ml Tf-TR for 2 h at 37°C to allow uptake. Cells were then washed and incubated with Neurobasal medium supplemented with B27, penicillin-streptomicin, glutamine, fungizone (all from Invitrogen), and 50 ng/ml NGF (Sigma). Twenty-seven hours later, neurons were fixed in 2% neutral buffered formalin for 30 min, washed with PBS, and kept at 4°C until use. Slides were mounted in VectaShield Mounting Medium with DAPI (Vector). Images were taken using a Leica SP2 AOBS SE (Leica Microsystems) confocal laser-scanning microscope. For the quantification of Tf-TR labeling in DRG neurons, a semiquantitative scale ranging from 1 to 5 was used as follows: 1, Tf-TR present in 100% of the neurites; 2, Tf-TR present in >50% of the neurites; 3, Tf-TR present in 50% of the neurites; 4, Tf-TR present in <50% of the neurites; 5, Tf-TR absent from neurites (i.e., only present in the cell body).

In vivo *analysis of retrograde transport using cholera toxin B.* The sciatic nerve of WT and TTR KO mice was exposed and transected at the midtight level; a solution of the retrogradely transported cholera toxin B subunit (0.5 mg/ml, List Biological) was applied to the proximal end of the transected sciatic nerve for 35 min. The skin was subsequently sutured and mice were allowed to recover for 72 h, after which the L4–6 DRG were collected and fixed in 4% neutral buffered formalin. To detect retrogradely labeled sensory neurons, serial 4-μm-thick DRG sections were cut and processed for anti-cholera toxin immunohistochemistry. Briefly, sections were blocked in blocking buffer (1% bovine serum albumin and 4% fetal bovine serum in PBS) for 30 min at 37°C and incubated with anti-cholera toxin antibody (Calbiochem; 1:1000) diluted in blocking buffer overnight at 4°C. Antigen visualization was performed with the biotin-extravidin-peroxidase kit (Sigma). For each animal, to determine the percentage of retrogradely labeled sensory neurons, the total number of DRG neurons, as well as the number of labeled DRG neurons presenting visible nuclei, were counted every 24 μm.

Analysis of TTR endocytosis by DRG neuron cultures. Primary cultures of DRG neurons were performed as described above (Lindsay, 1988). Cells were maintained for 96 h at 37°C. After this period, DRG neurons were supplemented with 300 μg/ml recombinant human TTR conjugated with Alexa 488 (hTTR-Alexa 488, produced as described above) for 3 h at 4°C or 37°C. When mentioned, neuronal cells were additionally incubated with 50 μg/ml transferrin conjugated with Texas red (Tf-TR, Invitrogen) or with 5 μg/ml cholera toxin subunit B conjugated with Alexa Fluor 647 (CT-B, Invitrogen). Cells were fixed in 2% neutral buffered formalin for 30 min, washed with PBS and kept at 4°C until immunostaining. For immunocytochemistry, DRG neurons were permeabilized with 0.2% Triton X-100 (Sigma) and were then incubated with 0.1% sodium borohydride (Sigma). For immunocytochemistry using the primary antibody rabbit anti-PGP 9.5 (1:500, Ab Serotec), coverslips were blocked in MOM IgG Blocking reagent (Vector) for 1 h at room temperature, and incubated with primary antibody diluted in MOM diluent (Vector) overnight at 4°C. Subsequently, incubation with the secondary antibody anti-rabbit IgG-Alexa 568 (1:1000, Invitrogen) diluted in MOM diluent was performed for 1 h at room temperature. Coverslips were mounted with VectaShield Mounting Medium containing DAPI (Vector), and images were taken using a Leica SP2 AOBS SE (Leica Microsystems) confocal laser scanning microscope.

Analysis of TTR endocytosis by transfected DRG neurons. Primary DRG neurons were obtained as already detailed. After DRG neuron isolation and prior plating, ~250,000 neurons were transfected with 10 μg of GFP-tagged Eps15 constructs (a kind gift from Dr. Benmerah, Institut Cochin, Paris, France), isolated using the Endofree Plasmid Maxi kit (Qiagen, Portugal). Transfection was performed in a Amaxa Nucleofector (Amaxa Biosystems), using program A-033 and the Mouse Neuron Nucleofector kit (Amaxa Biosystems). Transfected cells were subsequently plated in 24-well plates at a density of ~30,000 cells/well. Cells were maintained for 48 h at 37°C after which they were supplemented with 300 μg/ml recombinant human TTR conjugated with Alexa 568 (hTTR-Alexa 568, produced similarly as described above for hTTR-Alexa 488) for 3 h at 37°C. Cells were then processed for PGP 9.5 immunocytochemistry, as described above, using as secondary antibody anti-rabbit IgG-Alexa 647 (1:1000, Invitrogen). For quantification of hTTR-Alexa 568 internalization, images of randomly selected PGP 9.5 labeled cells were taken along the *z*-axis in a Leica SP2 AOBS SE confocal laser scanning microscope, using the same laser intensity for all conditions. hTTR-Alexa 568 internalization in cells photographed in each of the conditions was then determined using the ImageJ software (http://rsbweb.nih.gov/ij/) and calculated as total brightness intensity in the 568 channel (expressed as arbitrary units)/cell.

Measurement of neurite outgrowth using TTR coupled to FluoSpheres. Recombinant WT TTR (2 mg) was covalently bound to 1 μm-diameter carboxylate- or amine-modified FluoSpheres (Invitrogen), using a carbodiimide cross-linking method, according to the manufacturer's instructions. FluoSpheres bound to TTR were washed three times with PBS

and the efficacy of the method was evaluated by running the three washes containing free uncoupled WT TTR in a 15% SDS polyacrylamide gel; >90% of WT TTR was bound to the FluoSpheres and no detectable free TTR was found within the last wash. PC12 cells (European Collection of Cell Cultures), a rat adrenal cell line with a neuronal-like phenotype, were grown in six-well plates in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). When cells reached 50% confluence, fetal bovine serum was withdrawn and cells were supplemented with either: (1) 10% of either WT or TTR KO mouse serum, (2) 10% TTR KO mouse serum containing 300 μg/ml recombinant WT TTR, (3) 10% of TTR KO mouse serum containing 0.12% of free FluoSpheres, (4) 10% of TTR KO mouse serum containing 300 μg/ml free recombinant WT TTR and 0.12% of free FluoSpheres, and (5) 10% of TTR KO mouse serum containing 300 μg/ml recombinant WT TTR coupled to 0.12% FluoSpheres. Fifty hours later, PC12 cells were fixed in 2% neutral buffered formalin for 30 min, washed with PBS and kept at 4°C until further analysis. Neurite size was determined from 50× magnified fields. At least 150 cells were analyzed for each condition. To check whether TTR was biologically active when coupled to FluoSpheres, its ability to bind T_4 was evaluated. Briefly, a 100 μl suspension of either free FluoSpheres (0.12%) or 8.25 μg/ml TTR coupled to FluoSpheres (0.12%) was incubated with 50,000 cpm of [^{125}I]T_4 (Perkin-Elmer) in triplicates overnight at 4°C. As an internal control to ascertain the specificity of T_4 binding to TTR, 100 μl suspension of the same samples were coincubated with 50,000 cpm of [^{125}I]T_4 and nonradioactive T_4 in molar excess. Subsequently, FluoSpheres were washed three times with PBS and radioactivity of the pellets, corresponding to T_4 bound to TTR, was counted in a gamma-spectrometer (Wallac).

Analysis of TTR endocytosis in DRG neurons blocked with sheep anti-rat megalin antibody. After DRG neuron isolation, cells were plated in 24-well plates at a density of ~10,000 cells/well. Cells were maintained for 96 h at 37°C after which they were preincubated for 2 h at 37°C with sheep anti-rat megalin (1:200, kindly provided by Dr. Pierre Verroust, CHU Saint Antoine, Paris, France) or nonimmune sheep serum (using an equal volume as the one used in the case of sheep anti-rat megalin). Subsequently, DRG neurons were supplemented with 300 μg/ml hTTR-Alexa 488 in the presence of sheep anti-rat megalin or nonimmune sheep serum (in the same conditions as used for the preincubation) for 3 h at 37°C. It is noteworthy that this anti-megalin antibody has been used previously to successfully inhibit megalin-dependent internalization (Klassen et al., 2004). DRG neurons were then processed for PGP 9.5 immunocytochemistry as described above using as secondary antibody anti-rabbit IgG-Alexa 568 (1:1000, Invitrogen). For quantification of hTTR-Alexa 488 internalization, images of randomly selected PGP 9.5 labeled cells were taken along the *z*-axis in a Leica SP2 AOBS SE confocal laser scanning microscope, using the same laser intensity for all conditions. The intensity of internalized hTTR-Alexa 488 in DRG neurons treated with either sheep anti-rat megalin or nonimmune sheep serum was then determined using the ImageJ software (http://rsbweb.nih.gov/ij/), as described above.

Measurement of neurite outgrowth in the presence of TTR and anti-rat megalin. TTR KO DRG neurons were maintained for 48 h at 37°C, after which DRG neurons were supplemented with (1) B27 or B27 containing either (2) 300 μg/ml recombinant TTR, (3) 300 μg/ml recombinant TTR and 200 μg/ml sheep anti-rat megalin (kindly provided by Dr. Pierre Verroust), (4) 200 μg/ml sheep anti-rat megalin, and (5) 300 μg/ml recombinant TTR and 200 μg/ml IgG (IgG, Sigma). As an additional control, in an independent experiment, DRG neurons were supplemented with B27 containing a volume of nonimmune sheep serum equal to the one of sheep anti-rat megalin used in condition (5). Fifty hours later, cells were fixed in 2% neutral buffered formalin for 30 min, washed with PBS and kept at 4°C until further analysis. Neurite size was determined from 50× magnified fields. At least 180 cells were analyzed for each condition.

Megalin immunohistochemistry and RT-PCR. For RT-PCR, total RNA from mouse DRG and kidney was isolated using Trizol (Invitrogen). cDNA was obtained using the Superscript II kit (Invitrogen). PCR was performed using the following sense and antisense primers: for mouse megalin, 5′-CCTTGCCAAACCCTCTGAAAAT-3′ and 5′-CACAAGGTTTGCGGTGTCTTTA-3′ and for mouse HPRT, 5′-GTAATGATCAGTCAACGGGGGAC-3′ and 5′-CCAGCAAGCTTGCAACCTTAACCA-3′. Ethidium bromide-stained gels were scanned using a Typhoon 8600 (Amersham). For immunohistochemistry using the sheep anti-megalin primary antibody (1:2000, kindly provided by Dr. Pierre Verroust), antigen unmasking was done by boiling 3× in 0.5 mM EDTA 10 mM Tris pH = 9.0 solution. Sections were blocked in blocking buffer (1% bovine serum albumin and 4% fetal bovine serum in PBS) for 30 min at 37°C and incubated with primary antibody diluted in blocking buffer overnight at 4°C. Antigen visualization was performed with the biotin-extravidin-peroxidase kit (Sigma). Slides were counterstained with hematoxylin (Merck).

Statistical analysis. Statistical analysis was performed using the Student's *t* test. Results were expressed as average ± SEM.

Results

TTR is detectable within the endoneurium of both control and crushed nerves

We recently determined that TTR enhances nerve regeneration (Fleming et al., 2007). Upon nerve injury, disruption of the BNB occurs, leading to the exposure of the nerve to plasma proteins. To verify the disruption of the BNB by nerve crush, ultrathin nerve sections were analyzed, 3 d after crush; blood residues were observed near the crush site (Fig. 1*A*). To check whether TTR was present in the nerve 3 d after crush, mouse TTR immunoblotting of crushed nerves from perfused WT mice was performed. As illustrated in Figure 1*B*, TTR is indeed present in the nerve after crush, in the course of regeneration. As positive and negative controls, CSF from WT mice (WT CSF) and nerve extracts from TTR KO mice (TTR KO nerve) were used, respectively (Fig. 1*B*).

To further unveil the presence of TTR in the nerve in the settings of nerve injury, we performed immunohistochemistry against mouse TTR (mTTR) in crushed nerves from WT mice. mTTR was readily detected within the nerve, along nerve fibers (Fig. 1*Ca*). To confirm the specificity of mTTR immunohistochemistry, three different negative controls were performed: mTTR immunohistochemistry of (1) TTR KO crushed nerves (Fig. 1*Cb*), (2) WT crushed nerves using the anti-mTTR primary antibody previously adsorbed with recombinant mTTR (Fig. 1*Cc*), and (3) WT crushed nerves in the absence of primary antibody (Fig. 1*Cd*). As expected, no reactivity was present in the negative controls. Considering the prompt detection of TTR in the crushed nerve, we followed by investigating whether TTR was detectable in the nerve under physiological conditions. mTTR immunohistochemistry of WT intact nerves revealed that TTR is readily detectable in the nerve (Fig. 1*Da*) with a similar immunostaining pattern as the one observed after nerve crush (Fig. 1*Ca*). The analysis of transverse sections of WT intact nerves stained for mTTR suggests that TTR is probably present in the extracellular matrix surrounding nerve fibers, as visualized by colocalization with brightfield images (Fig. 1*Db–d*).

To further understand how TTR gains access to the nerve after injury, hTTR-Alexa 488 was injected intravenously 1 d before nerve crush; 1 d after crush, nerves were collected and cryopreserved. Subsequently, crushed nerves were screened for green fluorescence. Interestingly, hTTR-Alexa 488 was found within the nerve, along fibers (Fig. 1*Eb*). The same pattern was found in WT and TTR KO nerves (data not shown). To verify that the green fluorescence corresponded to injected hTTR-Alexa 488, immunohistochemistry against human TTR (hTTR) was performed, showing that both signals colocalized (Fig. 1*Eb–d*), confirming that plasma TTR enters the sciatic nerve after crush.

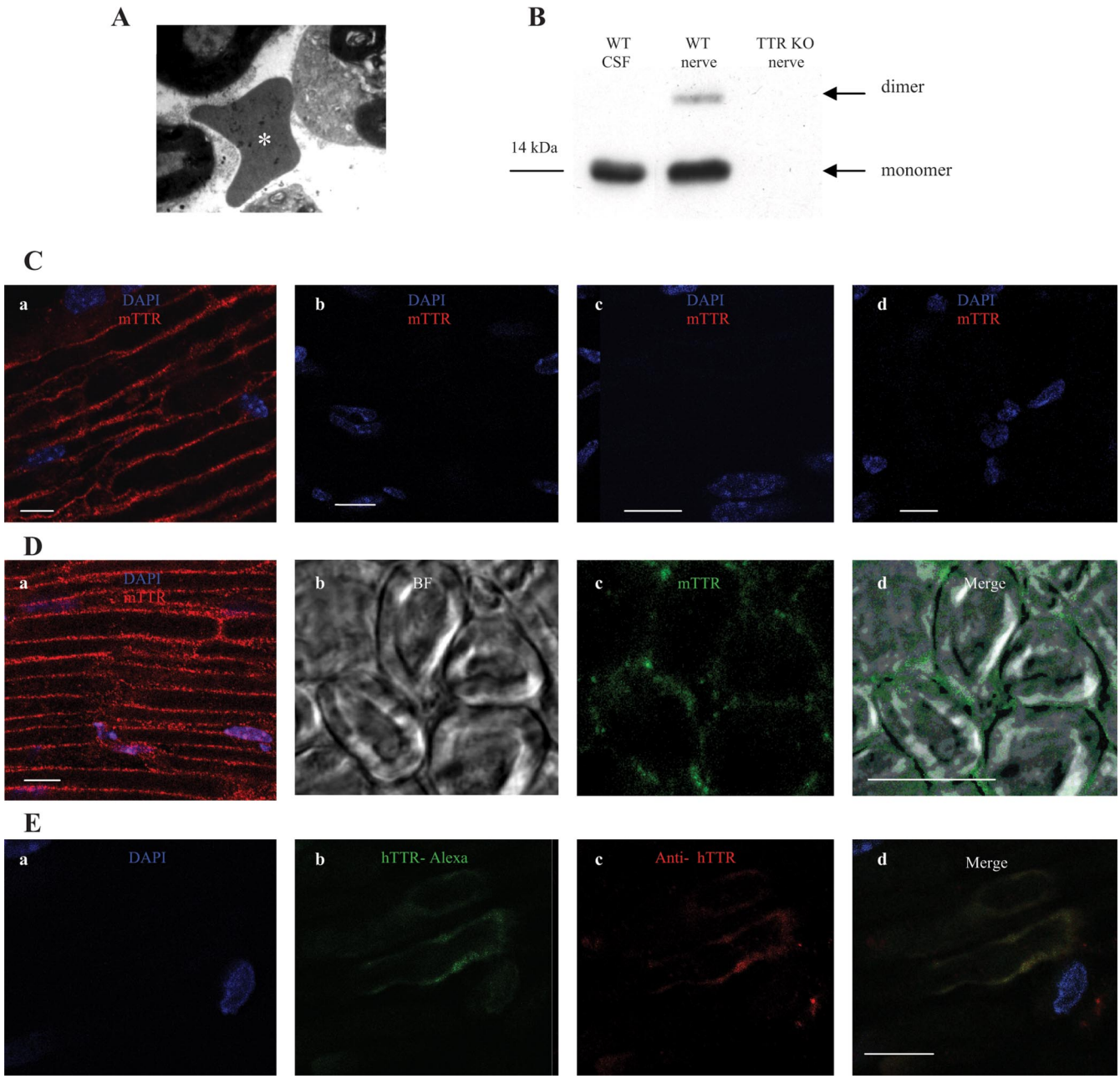

Figure 1. TTR presence in the nerve. ***A***, ***B***, TTR presence in the nerve 3 d after nerve crush as assessed by evaluation of ultrathin sections from a WT sciatic nerve near the crush site (asterisk highlights blood accumulation) (***A***), and anti-mouse TTR Western blot of WT mouse CSF (WT CSF), crushed nerves from WT (WT nerve), and TTR KO mice (TTR KO nerve) (TTR monomer and dimer are indicated by arrows) (***B***). ***C***, mTTR presence in the nerve after injury detected by immunohistochemistry in WT crushed nerves (***a***), TTR KO crushed nerves (***b***), and WT crushed nerves using anti-mTTR preincubated with recombinant mTTR (***c***), and in the absence of primary antibody (***d***); scale bars, 10 μm. ***D***, mTTR presence in intact nerves: (***a***) mTTR immunohistochemistry in a longitudinal section of an intact WT nerve and (***b–d***) mTTR immunohistochemistry of a transverse section of an intact WT nerve. BF, Brightfield. Scale bar, 10 μm. ***E***, Intravenously injected hTTR-Alexa 488 was found within WT crushed nerves along fibers: (***a***) DAPI immunostaining in blue, (***b***) hTTR-Alexa 488 in green, (***c***) hTTR immunostaining in red, and (***d***) merged image. Scale bar, 10 μm.

Local TTR delivery to the crushed nerve rescues the regeneration phenotype of TTR KO mice

In TTR KO mice, nerve regeneration is impaired: after 15 d of regeneration, the number of myelinated fibers is ~20% lower, whereas 30 d after injury, the density of unmyelinated fibers is ~40% decreased in this strain (Fleming et al., 2007). This impairment is recovered when TTR KO mice are backcrossed to mice expressing human TTR in neurons (Fleming et al., 2007). To determine if additionally, local TTR delivery to the injury site is sufficient to rescue the TTR KO phenotype, TTR was locally administrated to the crush site and mice were allowed to recover for either 15 or 30 d. Matrigel, the chosen vehicle, although containing other extracellular matrix components such as growth factors, is mainly constituted by laminin, that enhances nerve regeneration per se (Madison et al., 1985). To verify whether TTR released from Matrigel could be found at the nerve crush site, Matrigel supplemented with hTTR-Alexa 488 was initially used. As is shown in Figure 2*A*, hTTR-Alexa 488 could readily be found in the nerve crush site 1 d after Matrigel application (left); in nerves where free Alexa 488 was applied, no labeling was found, as expected (Fig. 2*A*, right).

Fifteen days after injury, the addition of Matrigel per se was

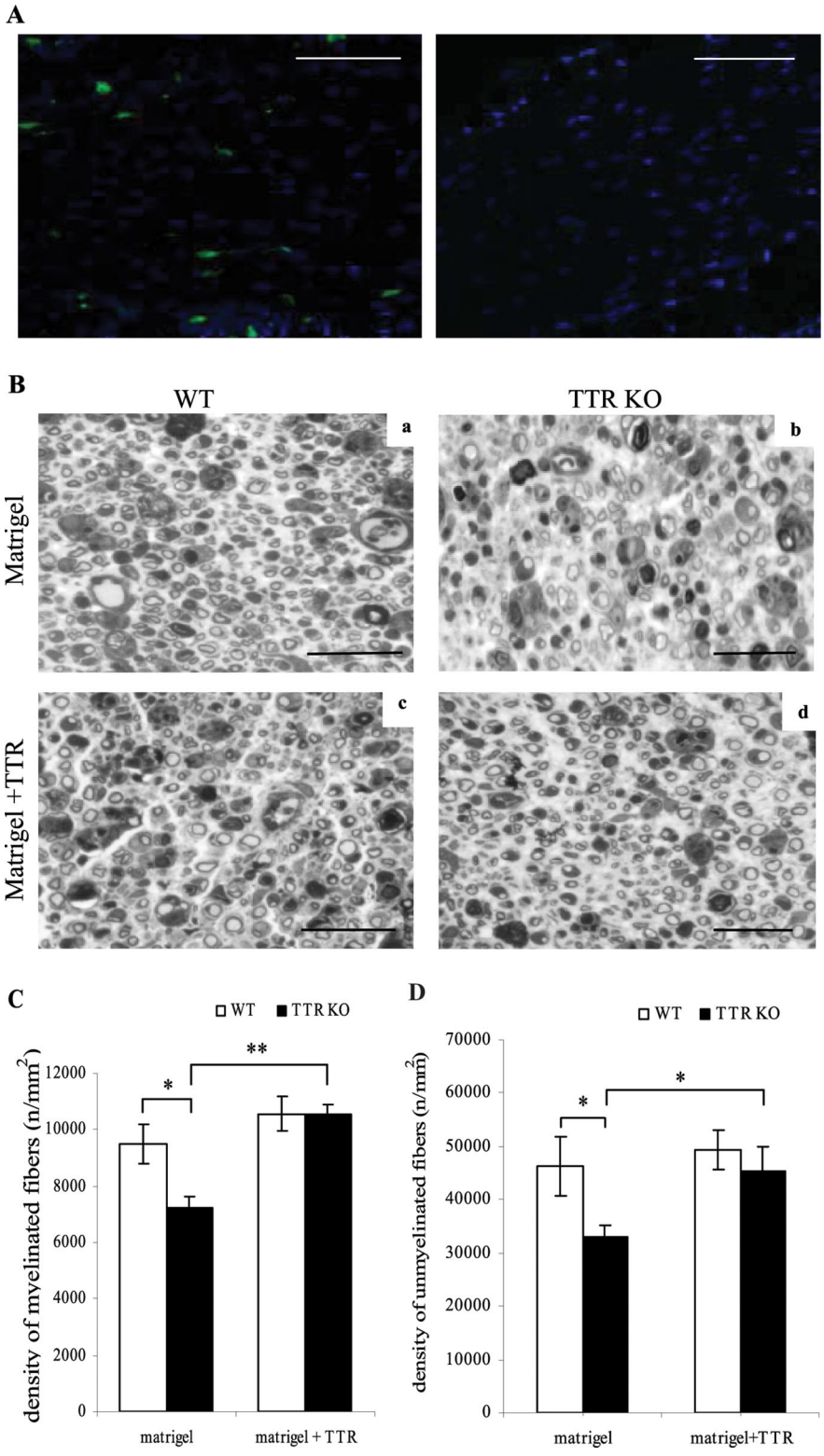

Figure 2. Local delivery of TTR to the crushed nerve. ***A***, Detection of green fluorescence in the sciatic nerve crush site 1 d after delivery of hTTR-Alexa 488 (left) or free Alexa 488 (right) in Matrigel. Scale bars, 100 μm; DAPI in blue. ***B***, Semithin sections of distal nerve stumps 15 d after nerve crush from WT mice (***a***, ***c***) and TTR KO mice (***b***, ***d***), in which Matrigel either alone (***a***, ***b***) or supplemented with TTR (***c***, ***d***) was added. Scale bars, 10 μm. ***C***, Corresponding density of myelinated fibers. WT: $n = 6$ and $n = 7$; TTR KO: $n = 5$ and $n = 7$, respectively, for each setting. ***D***, Density of unmyelinated fibers 30 d after nerve crush in WT and TTR KO mice, in which Matrigel either alone or supplemented with TTR was added. WT: $n = 6$ and $n = 5$; TTR KO: $n = 8$ and $n = 8$, respectively, for each setting; Matrigel alone (matrigel), Matrigel supplemented with TTR (matrigel+TTR). *$p < 0.05$, **$p < 0.01$.

not able to overcome the effect of lack of TTR, as TTR KO mice presented a 24% decreased density of myelinated fibers when compared with WT littermates (Fig. 2*B*,*C*). However, the addition of TTR to Matrigel was sufficient to rescue the regeneration delay of TTR KO mice, as their density of myelinated fibers reached WT levels (Fig. 2*B*,*C*).

Regarding unmyelinated fibers, at 15 d of regeneration no differences were found between the two strains (data not shown) which is in agreement with what was previously described at this time point after crush (Fleming et al., 2007). To further address whether TTR delivery to crushed nerves of WT and TTR KO mice could rescue the regeneration of unmyelinated fibers, their density was determined 30 d after injury. Similarly to what was observed for myelinated fibers, the addition of Matrigel per se was not able to overcome the lack of TTR, as 30 d after injury TTR KO mice presented a 30% decreased density of unmyelinated fibers when compared with WT littermates (Fig. 2*D*). However, the addition of TTR to Matrigel was able to rescue the regeneration impairment of TTR KO mice, as their density of unmyelinated fibers reached WT levels (Fig. 2*D*).

In TTR KO mice, after nerve crush, the Schwann cell response is unaffected while the inflammatory response reflects their delayed regeneration

Given that no effect of TTR on neuronal survival was found after nerve crush (Fleming et al., 2007), we assessed whether the delayed regeneration of TTR KO mice was associated with a differential response to injury by Schwann cells, by determining the percentage of proliferating and apoptotic cells in the nerve 5 d after crush. No differences in the number of BrdU-labeled cells were found between WT and TTR KO littermates (data not shown). Also, no differential cell survival was observed, as no differences were found between strains when the percentage of apoptotic cells was determined (data not shown). As no significant differences were found in the survival of either neurons or Schwann cells that could underlie the regeneration impairment in the absence of TTR, the persistence of macrophages was addressed in the distal stumps of regenerating nerves from WT and TTR KO mice 15, 30, and 75 d after nerve crush. Recruitment of macrophages is essential for rapid myelin clearance and therefore for regeneration to occur (Hirata and Kawabuchi, 2002); macrophages infiltrate the lesion site within 2 d and spread into the entire distal

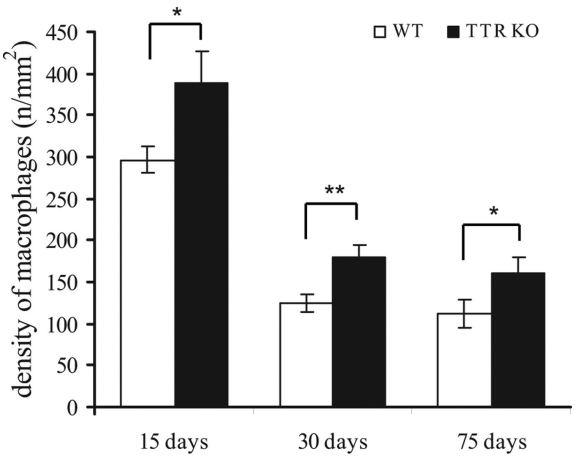

Figure 3. Macrophage response after nerve injury. Density of macrophages in regenerating nerves from WT and TTR KO mice 15, 30, and 75 d after nerve crush; $*p < 0.05$, $**p < 0.01$.

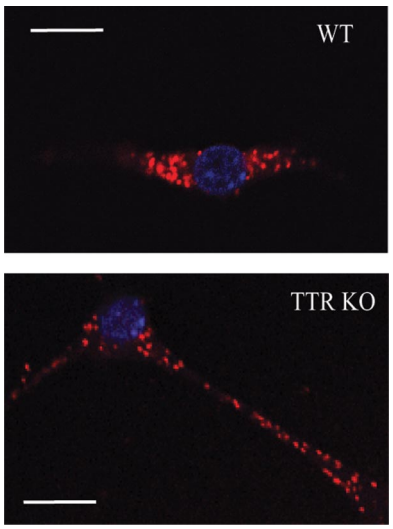

Figure 4. Retrograde transport in the presence and absence of TTR. Confocal images of WT and TTR KO DRG neurons 27 h after exposure to Tf-TR: in blue, DAPI staining, and in red, Tf-TR labeling. Scale bars, 10 μm.

stump after day 4 (Perry et al., 1987). At later time points, an increased macrophage number is recognized as an indicator that regeneration is compromised, as has been shown in other models of delayed axonal regeneration (Triolo et al., 2006). In both strains, as expected, the number of macrophages diminished with increased time of recovery (Fig. 3). Moreover, TTR KO mice presented a 30% increase in the density of macrophages after 15 d of regeneration, when compared with WT littermates; 30 and 75 d after nerve crush, this difference reached 45%. Such a persistence of macrophages in latter time points of regeneration is an additional indicator that myelin debris still exist in the nerves, i.e., that regeneration is delayed in TTR KO mice.

TTR KO mice present a compromised retrograde transport

To further understand the reason for the delayed regeneration in TTR KO mice, the capacity of WT and TTR KO axons to perform retrograde transport was assessed. In the case of impaired retrograde transport, the transmission of signals from the injury site to the cell body, that would allow the regrowth of fibers, might be compromised. $p75^{NTR}$ is a receptor that, upon binding to neurotrophins at axonal terminals, undergoes retrograde transport along the axon to the cell body (Curtis et al., 1995). Previous studies have shown that ligated nerves accumulate $p75^{NTR}$ in the distal side of the ligation, making this approach reliable for retrograde transport evaluation (Johnson et al., 1987, Taniuchi et al., 1988). Chronic constriction injury (CCI) was performed in WT ($n = 6$) and TTR KO ($n = 5$) mice and the accumulation of $p75^{NTR}$ in the distal side was determined 24 h after. TTR KO CCI nerves revealed a 26% decrease in the accumulation of $p75^{NTR}$ in the distal side of the ligation when compared with WT nerves ($p < 0.001$). To ensure that this decrease was not due to differential $p75^{NTR}$ expression, the levels of this protein were determined in WT and TTR KO intact nerves. No differences were observed between the two strains.

To further confirm that *in vivo* the absence of TTR is related to decreased levels of retrograde transport, WT ($n = 6$) and TTR KO ($n = 5$) sciatic nerves were retrogradely labeled with cholera toxin B. In agreement with the results obtained for the accumulation of $p75^{NTR}$, a decrease of ~30% was observed in the percentage of retrogradely labeled DRG neurons of TTR KO mice when compared with WT littermates ($p < 0.05$).

The relation between the absence of TTR and the lower levels of retrograde transport was additionally corroborated *in vitro*. For that, DRG neurons were cultivated and incubated with transferrin conjugated with Texas red (Tf-TR). Transferrin, a monomeric serum glycoprotein, binds iron for cell delivery through receptor-mediated endocytosis (Dautry-Varsat, 1986). When conjugated to Texas Red, transferrin functions as a tracer for retrograde transport (Liu et al., 2003). Twenty-seven hours after incubation, Tf-TR was only found in close proximity to the cell body in most WT DRG neurons (Fig. 4, top), while in the majority of TTR KO cells a more diffused labeling was found as Tf-TR was still present along neurites (Fig. 4, bottom). These differences in staining pattern are indicative of a slower retrograde transport in TTR KO DRG neurons. In fact, after quantification of the presence of Tf-TR in neurites and cell bodies, we determined that TTR KO DRG neurons presented a 20% decrease in the amount of retrogradely transported Tf-TR ($p < 0.001$). These *in vitro* findings correlate with the decreased accumulation of $p75^{NTR}$ in the distal portion of ligated TTR KO nerves, as well as with the decreased number of retrogradely labeled DRG neurons of TTR KO mice found *in vivo*, and suggest that lack of TTR is associated not only with decreased neurite outgrowth, but also with impaired retrograde transport.

In vitro, TTR is internalized by neurons through a clathrin-dependent mechanism

Since, *in vitro*, neurite outgrowth is increased by the presence of TTR (Fleming et al., 2007), we aimed at understanding how TTR action is exerted. For that, primary cultures of DRG neurons were incubated with hTTR-Alexa 488 for 3 h at 37°C. Confocal images along the *z*-axis showed that hTTR-Alexa 488 (in green) was internalized by DRG neurons. For visualization of cell bodies and neurites, the neuron specific protein PGP 9.5 (in red) was used. hTTR-Alexa 488 was prominently found in neurites (Fig. 5*A*, top panels, highlighted by arrows) but was also found within cell bodies (Fig. 5*A*, bottom panels, highlighted by arrows). Moreover, TTR internalization presented a punctate-like pattern (Fig. 5*A*), compatible with its presence within vesicles.

To establish whether TTR internalization was receptor-mediated, DRG neurons were incubated with hTTR-Alexa 488 for 3 h at 4°C, as at this temperature receptor-mediated endocytosis is inhibited. TTR internalization did not occur at 4°C, as

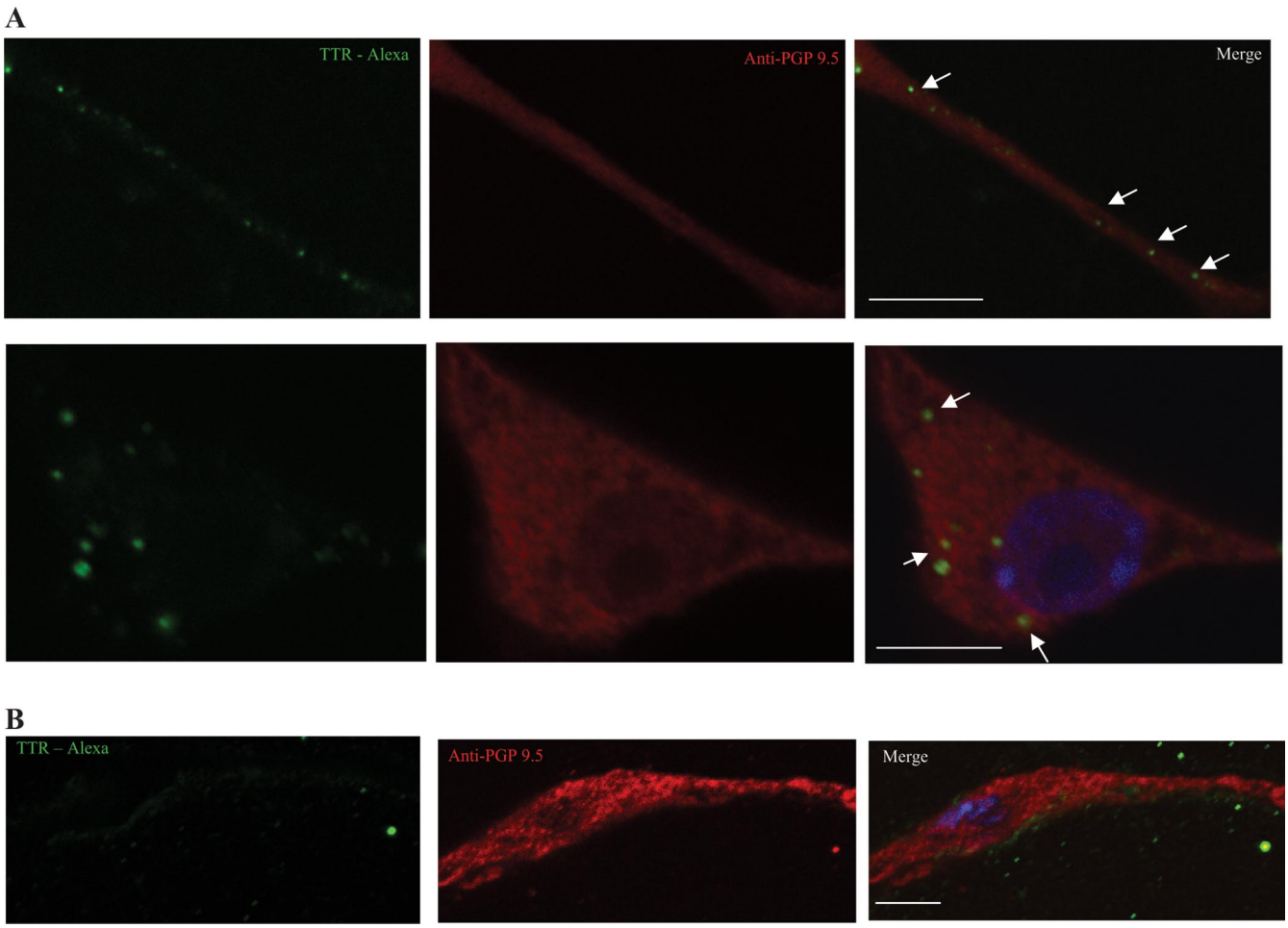

Figure 5. TTR internalization by DRG neurons. ***A***, ***B***, Confocal images of DRG neurons incubated with hTTR-Alexa 488 (in green) at 37°C (***A***, top shows a neurite and bottom shows a cell body) and at 4°C (***B***); anti-PGP 9.5 in red; internalized TTR highlighted by arrows. Scale bars, 10 μm.

hTTR-Alexa 488 was never found within DRG neurites or cell bodies (Fig. 5*B*), suggesting that TTR entry in neurons occurs through receptor-mediated endocytosis.

To further understand the mechanism through which TTR enters neurons, DRG neuron cultures were coincubated with hTTR-Alexa 488 and Tf-TR (in red), as transferrin endocytosis is known to be clathrin-dependent (Booth and Wilson, 1981). As shown in Figure 6*A*, hTTR-Alexa 488 vesicles partially colocalize with Tf-TR vesicles, suggesting that TTR endocytosis is clathrin-dependent. Reinforcing this finding, TTR did not colocalize with lipid rafts as shown by CT-B staining (in red) (Fig. 6*B*). In clathrin-coated pits, one of the major components, AP-2, is constitutively associated with Eps15, an accessory protein essential for early stages of clathrin-mediated endocytosis (Benmerah et al., 1998). To have additional proof that TTR endocytosis by DRG neurons is clathrin-dependent, GFP-tagged dominant negative constructs of Eps15 were transfected in DRG neurons. The two dominant-negative Eps15 mutants were: DIII and EΔ95/295 (Benmerah et al., 1998, 1999). As a control, DIIIΔ2 was used (Benmerah et al., 1998, 1999). The inhibition of transferrin uptake by both DIII and EΔ95/295 and the lack of effect of DIIIΔ2 are well described (Benmerah et al., 2000). Given its toxicity (A. Benmerah, personal communication), transfection of DRG neurons with the mutant EΔ95/295 resulted in high levels of cell death and as such only results obtained with the DIII dominant-negative Eps15 construct are described. In support of clathrin-mediated endocytosis, neurons transfected with the GFP-DIII construct displayed a reduced uptake of hTTR-Alexa 568 (Fig. 6*C*, top) when compared with DRG neurons transfected with the control construct GFP-DIIIΔ2 where TTR was taken up into the cytoplasm presenting the characteristic punctuate-like staining (Fig. 6*C*, bottom, arrowheads). Quantification of internalized hTTR-Alexa 568 using confocal images along the *z*-axis, revealed that in fact DRG neurons transfected with the GFP-DIII construct internalized 65% less hTTR-Alexa 568 than DRG neurons transfected with the control construct GFP-DIIIΔ2 ($p < 0.05$).

TTR internalization is required for neurite outgrowth enhancement

Similarly to DRG neurons, PC12 cells grown in the absence of TTR, i.e., with TTR KO serum, were previously shown to display a 30% decreased size of the longest neurite per cell, when compared with cells grown with WT serum (Fleming et al., 2007). Moreover, addition of TTR to TTR KO serum was able to rescue this phenotype. To understand whether TTR internalization is needed for the ability of TTR to enhance neurite outgrowth, additional experiments were performed where TTR KO serum was supplemented with TTR coupled to 1 μm-diameter polystyrene beads, FluoSpheres, which prevent TTR internalization due to their size. It is noteworthy that similar strategies using FluoSpheres were previously applied to address NGF internalization by neurons (Riccio et al., 1997; MacInnis and Campenot, 2002).

As a control, cells were also exposed to TTR KO serum supplemented with either FluoSpheres only or with FluoSpheres and free TTR. First, the efficacy of TTR binding to FluoSpheres was assessed: >90% of WT TTR was bound to the FluoSpheres and no detectable free TTR was found within the last wash (data not shown). Second, the biological activity of TTR coupled to FluoSpheres was assessed. TTR is a homotetrameric protein where the arrangement of the four subunits forms a central hydrophobic channel where T_4 binds (Blake et al., 1974). Given that the four TTR subunits need to be correctly structured so that T_4 binding is accomplished, this property of the protein was chosen to evaluate its structural integrity after conjugation to FluoSpheres. TTR coupled to FluoSpheres was efficient in binding T_4 (data not shown). As such, although it cannot be totally ruled out, it is unlikely that the results obtained with TTR conjugated to FluoSpheres might be the consequence of loss of function on sensory neurons caused by structural changes in the protein. Regarding neurite outgrowth, as previously described, absence of TTR in the cell culture medium (KO) was related to decreased size of the longest neurite, and addition of free TTR (KO + free TTR) was able to rescue this phenotype (Fig. 7). Addition of FluoSpheres to TTR KO serum did not alter neurite outgrowth (KO + FluoSpheres only). When PC12 cells were exposed to TTR KO serum supplemented with TTR coupled to FluoSpheres (KO + TTR coupled to FluoSpheres), neurite size was similar to the situation where cells were exposed to TTR KO serum alone (KO) (Fig. 7). Moreover, the addition of FluoSpheres and free TTR (KO + FluoSpheres + free TTR) rescued this phenotype, suggesting that the mechanism by which TTR enhances neurite outgrowth requires the internalization of the protein. To ascertain that the phenotype found was due to the inhibition of TTR internalization and not to the inactivation of specific residues of the protein, amine- and carboxylate-modified FluoSpheres were used in these assays. The results obtained were equivalent and independent of the type of FluoSpheres used (data not shown). This experiment was performed using PC12 cells as DRG neurons revealed to be sensitive to FluoSpheres which, under the conditions used, lead to the inhibition of neurite outgrowth independently of protein coupling.

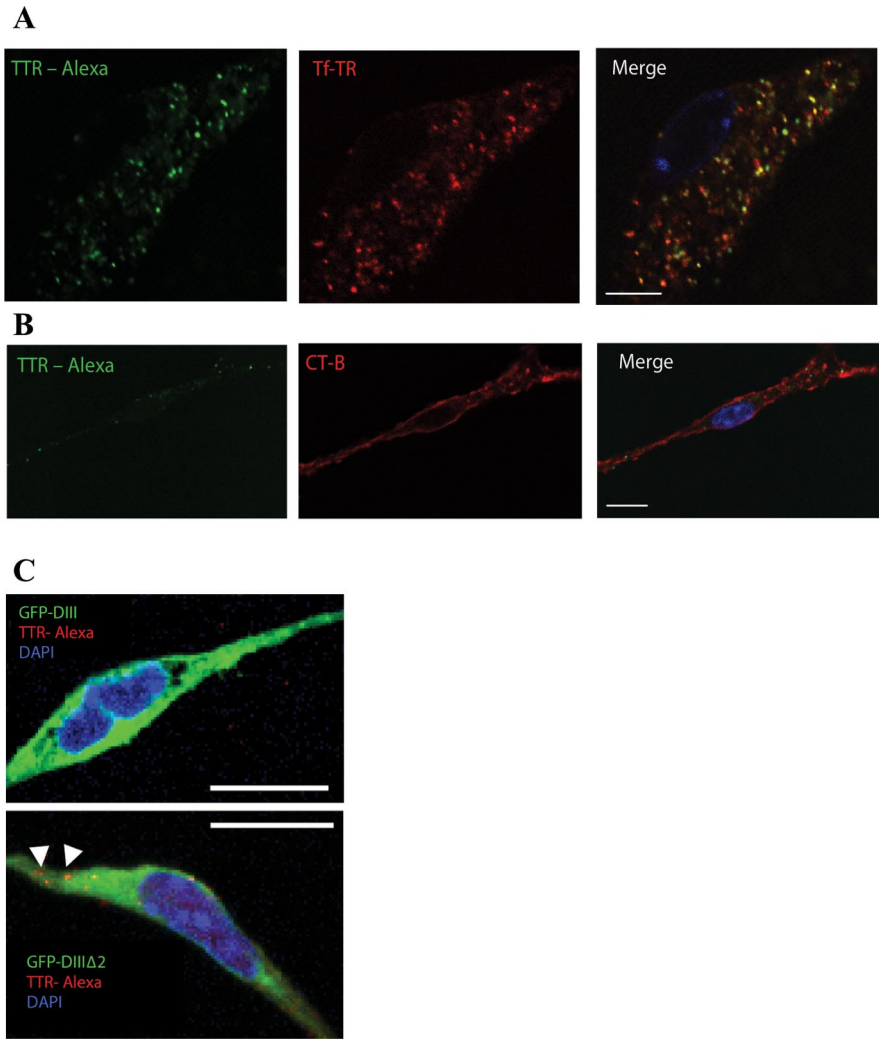

Figure 6. TTR internalization by DRG neurons is clathrin mediated. ***A***, Coincubation of DRG neurons with hTTR-Alexa 488 (green) and Tf-TR (red). ***B***, Coincubation of DRG neurons with hTTR-Alexa 488 (green) and CT-B (red). Scale bar, 10 μm. ***C***, *z*-axis stacking of hTTR-568 (red) uptake by DRG neurons transfected with either GFP-tagged DIII (green) (top) or DIIIΔ2 constructs (green) (bottom); DAPI staining in blue. Scale bar, 10 μm.

Megalin is expressed by DRG neurons and is necessary for TTR neuritogenic effect

Given that one endocytic TTR receptor, megalin, has been identified, being important for preventing TTR filtration through the glomerulus (Sousa et al., 2000) and since megalin was recently implicated in metallothionein uptake by neurons (Fitzgerald et al., 2007; Ambjørn et al., 2008), we hypothesized that megalin might be involved in the uptake of TTR. To verify whether DRG neurons express megalin, RT-PCR and immunohistochemistry were performed. Kidney was used as a positive control since megalin is abundantly expressed in this organ. As shown in Figure 8*A* and *B*, both methodologies demonstrate that megalin is expressed in DRG neurons. To demonstrate that a sheep anti-rat megalin antibody blocks TTR entrance in DRG neurons, similarly to what has been previously demonstrated for metallothionein uptake by kidney cells (Klassen et al., 2004), we performed the analysis of hTTR-Alexa 488 uptake in DRG neurons incubated with either nonimmune sheep serum or sheep anti-rat megalin. DRG neurons treated with the anti-megalin antibody (Fig. 8*C*, right) displayed less hTTR-Alexa 488 internalized when compared with DRG neurons incubated with nonimmune sheep serum (Fig. 8*C*, left). Quantification of internalized hTTR-Alexa 488 using confocal images along the *z*-axis, showed that DRG neurons where megalin was functionally blocked by the anti-megalin antibody revealed ~50% decreased hTTR-Alexa 488 entry relatively to DRG neurons incubated with nonimmune sheep serum ($p < 0.05$), similarly to what has previously been demonstrated for TTR uptake by kidney cells (Sousa et al., 2000). In light of these results, neurite outgrowth induced by TTR was measured after megalin blocking by anti-megalin. As previously described (Fleming et al., 2007), TTR KO DRG neurons grown in the pres-

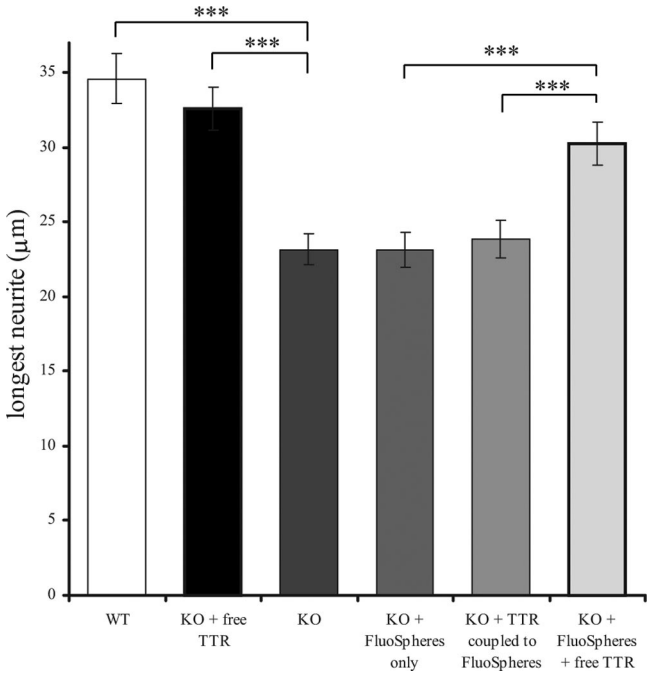

Figure 7. Neurite outgrowth in the presence of FluoSpheres. Average size of the longest neurite of PC12 cells after exposure to WT serum (WT), or to TTR KO serum alone (KO), or supplemented with either free TTR (KO + free TTR), FluoSpheres (KO + FluoSpheres only), TTR coupled to FluoSpheres (KO + TTR coupled to FluoSpheres), or FluoSpheres and free TTR (KO + FluoSpheres + free TTR). ***$p < 0.001$.

ence of TTR (+TTR) presented increased size of the longest neurite when compared with cells grown in TTR-free medium (Fig. 8*D*). TTR action was not affected by the addition of IgG (+TTR + IgG) (Fig. 8*D*). However, when DRG neurons were coincubated with TTR and anti-megalin (+TTR + α megalin), TTR enhancement of neurite size was no longer observed (Fig. 8*D*), showing that TTR internalization by neurons, which is needed for the protein to exert its neuritogenic activity, is megalin-dependent. As a control, the anti-megalin antibody was added to the culture medium (+ α megalin) and no difference was observed relatively to cells grown with culture medium alone. As an additional control, sheep serum was added to TTR KO DRG neurons, and again no difference was found when compared with cells grown with culture medium alone.

In vivo, decreased megalin leads to decreased nerve regeneration and its action is TTR-dependent

To assess the influence of megalin in the action of TTR *in vivo*, nerve crush of WT, TTR KO, megalin heterozygous (MEG (+/−)) and TTR KO/MEG (+/−) animals was done and mice were allowed to recover for 15 d. This experiment was performed with megalin heterozygous instead of megalin KO mice as most of the latter animals die within minutes after birth (Willnow et al., 1996). We started by determining that MEG (+/−) mice expressed ~50% less megalin in the DRG when compared with WT littermates (Fig. 9*A*) ($p < 0.05$). MEG (+/−) mice, producing a reduced amount of the receptor in the DRG, presented a 28% decrease in the density of myelinated fibers 15 d after crush when compared with WT animals (Fig. 9*B*). As previously described (Fleming et al., 2007), TTR KO mice also presented a 30% decrease in the density of myelinated fibers (Fig. 9*B*), emphasizing the role of TTR in nerve regeneration, and suggesting that in the absence of TTR, other megalin ligands have no influence in the progression of regeneration in the sciatic nerve. These findings additionally suggest that megalin is an important player in the course of nerve regeneration as its partial absence is sufficient to impair TTR-mediated enhancement of this process. Supporting this hypothesis, TTR KO/MEG (+/−) animals presented a similar regeneration impairment to both TTR KO and MEG (+/−) mice (Fig. 9*B*).

Discussion

In the present work we describe the mechanism of TTR action in neurons by showing that its neuritogenic effect is dependent on megalin-mediated internalization. We previously demonstrated that TTR enhances and accelerates regeneration (Fleming et al., 2007): 15 d after injury, the total number of myelinated fibers is 20% decreased in TTR KO mice (reaching WT levels after 30 d of regeneration), whereas in the case of unmyelinated fibers, differences are observed later, at 30 d after injury, when mice lacking TTR show a 40% decrease. More importantly, these differences have consequences at the functional level as TTR KO mice present a decreased locomotor activity and motor nerve conduction velocity throughout regeneration, clearly demonstrating that TTR enhances and accelerates this process. Such an effect is relevant in the scenario of nerve regeneration as timely target innervation is crucial for regain of functional capacity. Here, the impact of TTR in nerve regeneration was further established through the demonstration that local TTR delivery was successful in abolishing the differences between WT and TTR KO mice. Regarding the cellular response to nerve injury, TTR KO mice present no significant differences in both Schwann cell proliferation and survival when compared with WT littermates, suggesting that these mechanisms are not responsible for their delayed regeneration. Moreover, after nerve crush, no differences in the g ratio between strains were reported (Fleming et al., 2007), ruling out an impairment in myelination. The fact that TTR KO mice present an increased number of macrophages in the nerve after injury is probably an indicator that regeneration is compromised and that the removal of debris is delayed. Regarding the neuronal response to injury, the absence of TTR is not related to increased neuronal death after nerve crush that could underlie the diminished number of axons undergoing regeneration, but is instead related to delayed axonal growth (Fleming et al., 2007). Additionally to a decreased axonal growth, our present results demonstrate that TTR KO axons have lower levels of retrograde transport both *in vitro* and *in vivo*. Being the transmission of signals to the cell body a key process in nerve regeneration, the compromised retrograde transport of TTR KO axons might be, at least in part, responsible for the delayed regenerative capacity of TTR KO mice and decreased neurite outgrowth in the absence of TTR.

Our results show additionally that WT TTR is readily detectable within the peripheral nerve which is in agreement with the previous demonstration of TTR presence in the endoneurial fluid of human and rat nerves (Saraiva et al., 1988). The fact that TTR is present in the nerve under physiological conditions, as is here shown, further elucidates why, when mutated, TTR preferentially deposits in FAP peripheral nerves. In recipients of FAP livers, TTR deposits were found within the nerve, suggesting that plasma TTR can cross the BNB (M. M. Sousa et al., 2004). Our studies using intravenous injection of hTTR-Alexa 488 show that plasma TTR is in fact able to enter intact nerves through the BNB, which is effective in slowing but not in preventing the entry of proteins into the endoneurium (Wadhwani and Rapoport, 1994). This is in agreement with previous studies showing that intravenously injected albumin was conspicuously found

throughout nerve connective tissue and in basement membranes surrounding nerve fibers (Allen and Kiernan, 1994), similarly to what we here show for TTR. On the other hand, when an injury takes place, plasma TTR has its entrance in the nervous tissue facilitated, as the BNB is disrupted. In summary, our data further substantiates that *in vivo* TTR is present in the nerve and, as such, is able to contribute to the enhancement of nerve regeneration and neurite outgrowth in the settings of nerve injury.

In vitro, DRG neurons were able to internalize TTR by a clathrin-dependent endocytic process. The biological significance of TTR internalization was confirmed by the fact that the enhancement of neurite outgrowth by TTR was only possible when free TTR was used, being abolished when the protein was prevented from being internalized. Two endocytic TTR-related receptors have been described, namely megalin (Sousa et al., 2000) and an unidentified receptor-associated protein (RAP)-sensitive receptor (Sousa and Saraiva, 2001). Megalin, a member of the LDL receptor family, is involved in receptor-mediated endocytosis in clathrin-coated pits of a wide range of ligands, such as albumin (Zhai et al., 2000), and was described as being important for preventing TTR filtration through the glomerulus (Sousa et al., 2000). Later, it was shown that TTR internalization by liver cells is associated to lipoprotein metabolism, and that an unidentified RAP-sensitive receptor mediates TTR uptake (Sousa and Saraiva, 2001). Regarding the nervous system, megalin has been described as an important protein for the development of the forebrain (Willnow et al., 1996; Spoelgen et al., 2005) and spinal cord (Wicher and Aldskogius, 2008). Furthermore, megalin was thought to be expressed exclusively by epithelial cells; however, very recently, several reports have shown that megalin is expressed by other cell types, namely oligodendrocytes (Wicher et al., 2006), astrocytes (Bento-Abreu et al., 2008), and neurons, including retinal ganglion cells (Fitzgerald et al., 2007), cortical neurons (Chung et al., 2008), and cerebellar granule neurons (Ambjørn et al., 2008). We now show that DRG neurons also express megalin and that TTR neuritogenic activity depends on its internalization by this receptor. Interestingly, it was recently reported that metallothionein stimulation of neurite outgrowth in retinal ganglion cells (Fitzgerald et al., 2007) and cerebellar granule neurons (Ambjørn et al., 2008) is mediated by megalin. Considering that TTR interacts with metallothionein (Gonçalves et al., 2008) and that the present work demonstrates that TTR neuritogenic effect is megalin dependent, the relationship between these molecules and neurite outgrowth should be further studied. The present work also reveals that, *in vivo*, decreased levels of megalin lead to decreased nerve regeneration, further substantiating the importance of this receptor in the nervous system and particularly in the course of nerve regeneration. Although MEG (+/−) mutants are generally described as lacking a major phenotype, a semidominant effect through haplo-insufficiency resulting in decreased levels of megalin has been shown to cause progressive hearing loss in these animals (König et al., 2008). In support of diminished levels of megalin in MEG (+/−) mice, these were shown to present transferrin excretion in the urine, in contrast to WT mice (Kozyraki et al., 2001). Here we show that similarly to the latter tissues, MEG (+/−) DRG have a decreased expression of the receptor when compared with WT littermates. The importance of megalin in the nerve is supported by the fact that its partial absence is sufficient to impair TTR-mediated enhancement of nerve regeneration. Our data additionally suggests that, in the case of sciatic nerve regeneration, other megalin ligands have no influence, as TTR KO mice have a similar decrease in nerve regeneration as MEG (+/−) mice. This further suggests that megalin and TTR may act in the same pathway. It is worth mentioning that it is possible that additionally, TTR may generate its effect via signal transduction pathways ac-

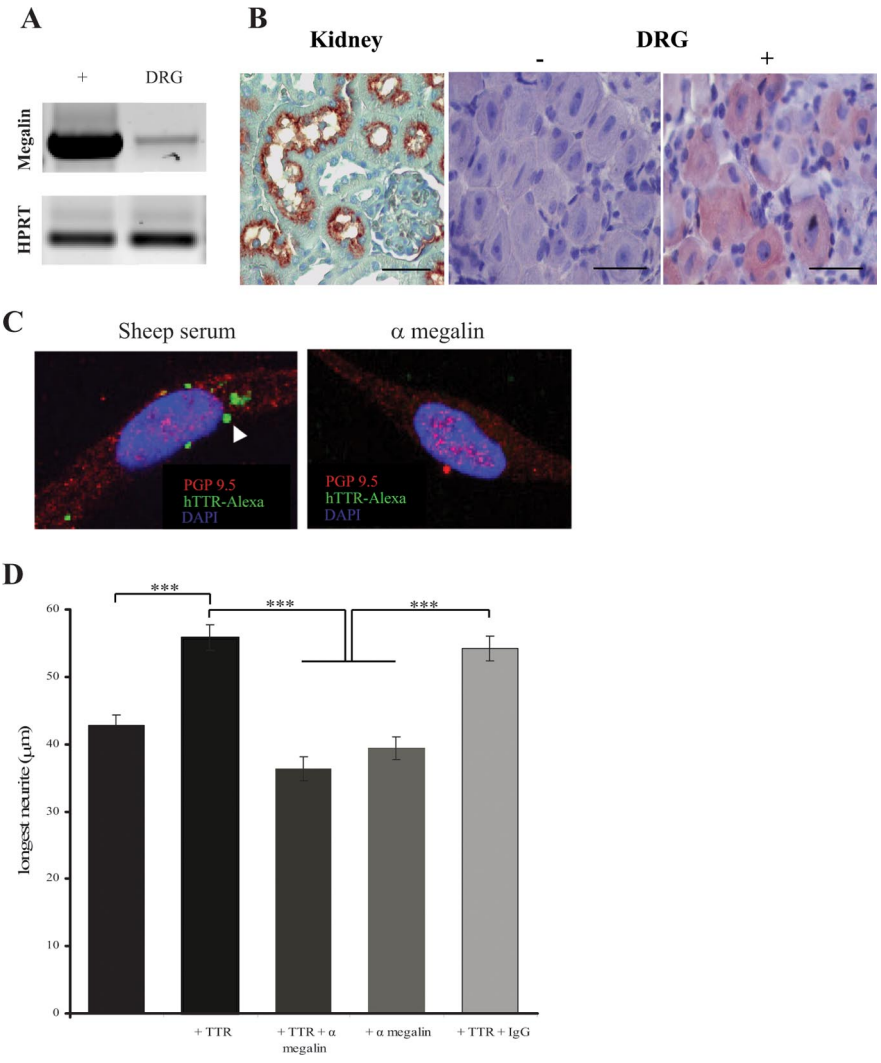

Figure 8. TTR internalization by DRG neurons is megalin mediated. ***A***, Megalin and HPRT RT-PCR of kidney (+) and DRG. ***B***, Megalin immunohistochemistry of kidney (left), and DRG in the absence (−, middle) and presence (+, right) of primary antibody. Scale bar, 5 μm. ***C***, *z*-axis stacking of hTTR-Alexa 488 (green) uptake by DRG neurons preincubated with either nonimmune sheep serum (sheep serum, left) or sheep anti-rat megalin (α megalin, right); red: anti PGP 9.5 staining, blue: DAPI. ***D***, Average size of the longest neurite of TTR KO DRG neurons cells after exposure to B27 or B27 supplemented with TTR (+TTR), with TTR and anti-megalin (+TTR + α megalin), with anti-megalin (+α megalin), or with TTR and IgG (+TTR + IgG). ***$p < 0.001$.

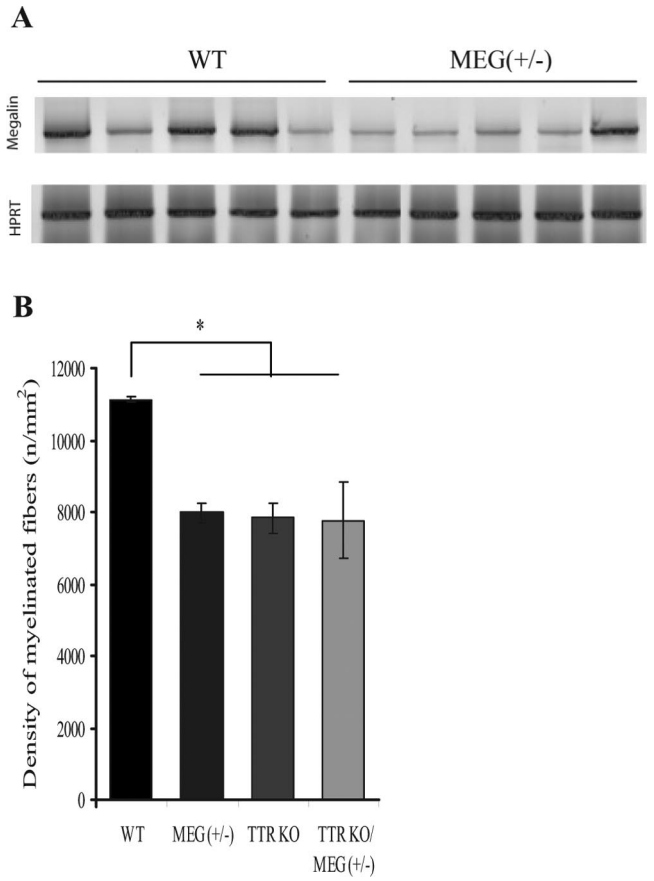

Figure 9. Decreased megalin levels lead to decreased nerve regeneration. ***A***, RT-PCR analysis of megalin and HPRT expression in MEG(+/−) and WT mouse DRG. ***B***, Morphometric analysis of sciatic nerves from WT, TTR KO, MEG(+/−), and TTR KO/MEG(+/−) mice. Density of myelinated fibers 15 d after nerve crush. *$p < 0.05$.

tivated by the NpxY motifs of the cytoplasmic tail of megalin, which interact with signaling molecules involved in the regulation of endocytosis (Qiu et al., 2006). It is also noteworthy that megalin is part of a ligand-dependent signaling pathway by enzymatic processing, linking receptor-mediated endocytosis with cell signaling (Zou et al., 2004).

In conclusion, our work further establishes the mechanism of TTR action in the nerve by showing that it has a direct effect on neurons, as its absence leads to impaired retrograde transport and decreased axonal growth. Also, we show that TTR effect in neurite outgrowth and nerve regeneration is mediated by megalin-dependent internalization. Finally, the relevance of TTR presence in the nerve may underlie its preferential deposition, when mutated, in the PNS of FAP patients.

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Considering the knowledge that I have acquired since the publication of this paper, I propose several experiments that could be performed in order to understand the molecular mechanisms through which TTR increases axonal growth.

- Determine whether in the adult, the effect of TTR is restricted to DRG neurons

The role of TTR in the PNS has been extensively studied due to its involvement in familial amyloid polyneuropathy (FAP), a neuropathy characterized by the deposition of TTR amyloid fibrils in the PNS (Sousa and Saraiva, 2003). The preference for TTR accumulation in peripheral nerves led to the hypothesis that WT TTR may play a role in PNS physiology. TTR is important for axonal growth since TTR KO DRG neurons have decreased neurite outgrowth, and TTR KO mice present impaired retrograde transport and delayed axonal regeneration (Fleming et al., 2009). This novel role of TTR in nerve physiology raises the question of whether TTR might also be important in the CNS. In this respect, it is important to note that TTR is one of the most abundant proteins in the cerebrospinal fluid (CSF). As such, it would be interesting to know if TTR is also important for axonal elongation of CNS neurons, particularly in hippocampal neurons, as the presence and expression of TTR by this neuronal subtype has been suggested (Li et al., 2011).

- Dissect the involvement of TTR in axonal transport

One of the most important features in TTR KO mice is that they present impairment in retrograde axonal transport as determined by retrograde labeling with cholera toxin and by analysis of transport of $p75^{NTR}$. Nevertheless, very little is known about the mechanism by which TTR interferes with axonal transport. It is not even known if the defects in axonal transport are exclusive to retrograde transport or whether TTR KO mice also have defects in anterograde transport. To investigate this hypothesis, the accumulation of an anterogradely transported protein like amyloid precursor protein (APP) (Cavalli et al., 2005) could be studied in the proximal stump of TTR KO mice following ligation of the sciatic nerve. Moreover, different components of the axonal transport could be tested *in vitro*, namely organelles, vesicles and proteins to identify which components are

impaired in TTR KO mice. To assess organelle transport, mitochondria labeling could be performed with mitoTracker (Invitrogen); for vesicles, fluorescently labeled synaptophysin or APP transfected neurons could be used; and for protein transport studies with, fluorescently labeled tubulin could be performed. These experiments could further disclose the involvement of TTR in axonal transport.

Additionally, whether the defects in axonal transport are a direct cause of the absence of TTR or are a secondary result from the lack of this protein should be further investigated.

Besides, at the molecular level many questions can be raised. Are microtubules correctly assembled in axons of TTR KO mice? To evaluate these issues, microtubules could be extracted from nerves of TTR KO mice and WT littermates and the ratio of microtubule/tubulin monomer could be established.

- Identify downstream targets of TTR that could mediate axonal growth

The dissection of downstream targets of TTR might unravel novel regeneration enhancers that could be modulated to increase nerve regeneration. Since TTR was found to be important in axonal growth of DRG neurons (Fleming et al., 2009), a screening could be done on DRG neurons, from naïve and PNS injured TTR KO mice and WT littermates. One of two approaches could be performed. Either microarray to evaluate different patterns of gene expression or a proteomic approach such as iTRAQ or 2D gels coupled to mass spectrometry. The proteins/genes differentially regulated should then be validated. Moreover, since TTR has also been described as a protease (Liz et al., 2012), and since its activity is important for its ability to induce neurite outgrowth (Liz et al., 2009), it would be interesting to know if the identified proteins are TTR substrates.

- Determine whether in FAP, loss of function (as an axonal regeneration enhancer) of mutated TTR aggregates/fibrils also contributes to neuropathology.

In FAP, there is the accumulation of mutated TTR in peripheral nerves that leads to neurodegeneration. Neurodegeneration is attributed to the toxicity of TTR aggregates (Sousa and Saraiva, 2003). Nevertheless, it cannot be excluded that

loss of function of TTR fibrils, may also contribute to neuropathology. In particular, given that TTR is important in retrograde transport, impairment in this function may contribute to pathology. In fact, defects in axonal transport have been associated to neurodegenerative diseases (Chevalier-Larsen and Holzbaur, 2006). This could be addressed by using animal models of FAP to check whether these present defects in axonal transport. It would be interesting to know if these putative defects precede amyloid deposition and neurodegeneration.

Chapter I

Cholesterol and sphingomyelin are axon regeneration inhibitors that can be counteracted by cyclodextrin delivery

Abbreviated title: Myelin lipids modulate axonal regeneration

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Abstract

Lack of axonal regeneration following spinal cord injury has been mainly ascribed to the inhibitory environment of the injury site i.e., to chondroitin sulphate proteoglycans and myelin-associated inhibitors. Here, we used shiverer mice to assess axonal regeneration following spinal cord injury in the presence of myelin-associated inhibitors and chondroitin sulphate proteoglycans, but in the absence of compact myelin. Although *in vitro* shiverer neurons displayed a similar intrinsic neurite outgrowth to wild-type neurons, *in vivo*, shiverer fibers had increased regenerative ability, suggesting that the wild-type spinal cord contains additional inhibitors besides myelin-associated inhibitors and chondroitin sulphate proteoglycans. Our data shows that besides myelin protein, myelin lipids are highly inhibitory for neurite outgrowth and demonstrates that this inhibitory effect is released in the shiverer spinal cord given its decreased lipid content. Specifically, we identified cholesterol and sphingomyelin as novel myelin-associated inhibitors with activity in multiple neuron types. We further demonstrated the inhibitory action of cholesterol and sphingomyelin *in vivo*, by showing that delivery of 2-hydroxypropyl-β-cyclodextrin, a drug that reduces the levels of lipids in the injury site, leads to increased axonal regeneration following spinal cord injury. In summary, our work shows that myelin lipids are important modulators of axonal regeneration that should be considered together with protein myelin-associated inhibitors as critical targets in strategies aiming at improving axonal growth following injury. In this respect, our study provides the initial preclinical data needed to evaluate the possible use of 2-hydroxypropyl-β-cyclodextrin in clinical trials with spinal cord injury patients.

Introduction

The inability of adult vertebrate CNS axons to regenerate is seen as a consequence of the highly inhibitory environment at the injury site (Silver and Miller, 2004), and of the failure in activating a cell-intrinsic program leading to the expression of regeneration-associated genes (Sun and He, 2010). Upon CNS injury, the glial scar functions as an inhibitory barrier composed of multiple components, generally divided in 3 categories: myelin associated inhibitors (MAIs), namely Nogo, myelin associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) (Lee and Zheng, 2012); canonical axon guidance molecules, such as semaphorin 3A, ephrin B3, netrin-1 and repulsive guidance molecule A (RGMa) (Giger et al., 2010); and chondroitin sulfate proteoglycans (CSPGs), produced by astrocytes (Jones et al., 2003).

Studies from different groups using triple knockout mice for MAG, Nogo and OMgp produced conflicting results ranging from limited (Lee et al., 2010) to extensive regeneration abilities (Cafferty et al., 2010). Although the field has largely concentrated on MAIs, other inhibitors play important roles *in vivo*: blocking CSPG or RGMa or deleting ephrinB3 increases axonal regeneration following spinal cord injury (SCI) (Bradbury et al., 2002; Hata et al., 2006; Duffy et al., 2012). As such, the wide variety of inhibitors present in the spinal cord milieu is thought to underlie the absence of a robust axonal regeneration when blocking either single or a limited combination of inhibitors. Despite the structural differences, several MAIs share receptors and an inhibitory mechanism dependent on RhoA/ROCK activation (Yiu and He, 2006), as demonstrated mainly through the use of RhoA/ROCK inhibitors (Kubo and Yamashita, 2007).

To further explore the importance of myelin in the inhibition of axonal regeneration, we used shiverer (shi) mice which lack myelin basic protein (MBP), a key player in myelin compaction in the CNS (Boggs, 2006). In the shi CNS, the absence of MBP results in a complete lack of compact myelin (Rosenbluth, 1980) which is accompanied by a severe phenotype comprising shivering, convulsions and early death. Here we show that in shi mice, despite the presence of canonical MAIs and of axonal abnormalities generally related to decreased regeneration capacity, CNS axons have an increased ability to regenerate through the spinal cord glial scar. This increase in regeneration was ascribed to a decreased abundance of myelin lipids, namely cholesterol and sphingomyelin. Moreover we

show that delivery of 2-hydroxypropyl-β-cyclodextrin (HPβCD), reduces the levels of cholesterol and sphingomyelin in the SCI site, leading to increased axonal regeneration. In summary, our work demonstrates that myelin lipids are important modulators of axonal regeneration that can be targeted through HPβCD delivery.

Materials and methods

Animals. Mice were handled according to European Union and National rules. All procedures were approved by the IBMC Ethics Committee and by the Portuguese General Veterinarian Board. Six week-old-WT and shi (Jackson laboratories) littermates of either sex, on a Swiss Webster:C3HeB/Fe5 background, were obtained from heterozygous breeding pairs. For drug-delivery studies 8 weeks-old C57BL/6 mice of either sex were used.

Electron microscopy. The thoracic spinal cord of WT and shi littermates (11 weeks-old) was embedded in Epon. Semi-thin 1µm-thick sections were stained with p-phenylene-diamine to visualize myelin and white matter tracts. 60nm-thick sections of the dorsal funiculus were stained with uranyl acetate and lead citrate and examined in a JEOL JEM-1400 transmission electron microscope.

Western blotting. Twenty five µg of thoracic spinal cord protein from naïve (6 weeks-old) or injured (11 weeks-old) WT and shi littermates were separated in 3-8% Tris-Acetate gels (Bio-Rad) and transferred to nitrocellulose. Antibodies used: mouse anti-MAG (Dr. Richard Quarles, NINDS, Bethesda, 1:1,000), rat anti-OMgp (R&D Systems, 1:250), rabbit anti-Nogo-A (Dr. Stephen Strittmatter, Yale University, New Haven, 1:10,000), rabbit anti-Ephrin B3 (Santa Cruz Biotechnology, 1:200), rabbit anti-RGMa (Immuno-Biological Laboratories, 1:200) and mouse anti-GAPDH (Santa Cruz Biotechnology, 1:2,000).

MAG and Nogo immunohistochemistry. Paraffin-embedded spinal cords from WT and shi littermates (6 weeks-old) were used for immunohistochemistry against MAG (1:500) or Nogo-A (1:5,000) using ABC Vectastain and DAB (Vector labs).

Spinal cord injury (SCI). Animals were anesthetized with ketamine (75mg/kg)/medetomidine (1mg/kg) and a laminectomy was performed at the T9 level. Complete transection, dorsal hemisection or left lateral hemisection were done using a micro feather ophthalmic scalpel. Analgesia was performed for 72h with buprenorphine (0.08mg/kg).

Assessment of the lesion area following SCI. Five weeks after complete SCI, 10µm sagittal spinal cord cryosections were immunostained for CSPG (Sigma-Aldrich, 1:200) and GFAP (DAKO, 1:500). Collagen was visualized with Masson trichrome staining (Sigma-Aldrich). The injury area was measured using Photoshop CS3.

Regeneration of dorsal column fibers. Animals with dorsal hemisection were allowed to recover for 4 weeks. Four days before sacrifice, animals were anesthetized as described above and 2µl of 1% cholera toxin B (CT-B, List Biologicals) were injected in the left sciatic nerve. Fifty µm sagittal free floating sections were immunostained for CT-B (List Biologicals, 1:30,000). From each animal, the section displaying the highest number of regenerating axons was selected. Axonal regeneration was quantified by counting the number of CT-B labeled axons within the glial scar and by measuring the length of the longest fiber found rostrally to the injury border. All quantifications of axonal regeneration were performed with the researcher blinded for genotype and experimental group.

Regeneration of raphespinal fibers. Five weeks after complete spinal cord transection, free floating sections were immunostained for serotonin (5-HT) (1:20,000, Immunostar). Only animals where a complete injury was present in all sections were analyzed. Axonal regeneration was quantified by counting 5-HT positive fibers caudally to the injury site. Raphespinal fiber sprouting was assessed 4 weeks after lateral spinal cord hemisection by 5-HT immunostaining in cross-sections of the lumbar enlargement. 5-HT immunoreactivity was quantified in 4 sections/animal using FeatureJ software. Compensatory sprouting was quantified by 5-HT immunoreactivity in the ipsilateral ventral horn. For each genotype, 5-HT immunoreactivity was normalized against 5-HT immunoreactivity in the naïve spinal cord. All quantifications of axonal regeneration were performed with the researcher blinded for genotype.

Crude membrane isolation. Crude membranes were isolated from the spinal cord of 6 weeks old WT and shi littermates, as described (Shen et al., 1998). Briefly, tissues were homogenized in 0.32M sucrose, and nuclei removed by centrifugation at 500g for 30 min. Membranes were pelleted by centrifugation at 100,000g for 1 h, resuspended in H2O and protein concentration was determined.

Myelin isolation. Myelin was isolated from the spinal cord of 16 weeks-old WT mice as detailed (Norton and Poduslo, 1973). Briefly, the tissue was homogenized in 0.32M sucrose and after centrifugation at 900g, the post-nuclear supernatant was collected and carefully overlaid on an ultracentrifuge tube containing a 0.85M sucrose solution on top of a 50% (w/v) sucrose cushion. After centrifugation for 1

h at 37,000g at 4ºC, the interphase between sucrose solutions was transferred to a new ultracentrifuge tube. Two rounds of osmotic shocks were performed by adding ice-cold water and centrifugation at 20,000g. The final myelin pellet was stored at -80ºC until further use. Before use, myelin was sonicated and the protein concentration determined.

Lipid isolation. Lipids were isolated from myelin and spinal cord extracts by a modified Folch two solvent system (Mirza et al., 2007). Briefly, 150µg of protein extract (from either myelin or total spinal cord extract) were dissolved in methanol:water (1:1) and lipids were separated by liquid extraction with chloroform (Merck). The chloroform layer containing the lipids was then dried under nitrogen stream and stored at -20˚C. Absence of proteins in the lipid fraction was confirmed by western blotting against Nogo, MAG, Ephrin B3 and RGMa. The aqueous layer containing the protein sample was precipitated with 0.2M perchloric acid following Folch extraction. Following centrifugation the protein was resuspended in H2O, sonicated and its concentration was determined.

Lipid extraction and thin layer chromatography. Thoracic spinal cords from WT and shi littermates were collected and lipids were extracted as described (Folch et al., 1957). The spinal cord injury site and a rostral uninjured region of WT spinal cord treated with artificial CSF (aCSF) or HPβCD was also collected for lipid extraction. Acidic and neutral lipids were further separated by reverse-phase chromatography (Vance and Sweeley, 1967; Seyfried et al., 1978; Rodrigues et al., 2009). All lipids were analyzed by high-performance thin-layer chromatography (HPTLC) as detailed (Rodrigues et al., 2009) alongside with lipid standards. The lipids analyzed were: ceramide (Cer), phosphatidylserine (PS), cholesterol (CO), sulfatide (GS), galactocerebroside (GalCer), phosphatidylcholine (PC), triglycerides (TG), sphingomyelin (SpH), cholesteryl esters (CE), phosphatidylethanolamine (PE), globotetrahexosylceramide (Gb4), lactocerebroside (Lac), phosphatidylinositol (PI) and GM1 ganglioside (GM1).

Neuron cultures and inhibition assays. Primary cultures of DRG neurons from P6 mice were performed as described (Miranda et al., 2011). Glass coverslips were coated with poly-L-lysine (20µg/mL) and laminin (0.5µg/mL) followed by crude membranes (0.1µg protein), myelin (0.5µg protein), myelin protein fraction (0.5µg), MBP (Upstate, 0.9µg) or total protein from spinal cord extracts (3µg). To

test the effect of lipids, the myelin lipid fraction (corresponding to 0.5µg of protein) and lipids extracted from the spinal cord of either WT or shi mice (corresponding to 2µg of protein) were used. Coverslips were coated with poly-L-lysine followed by lipids in chloroform/methanol/water (2:1:0.1) and laminin (0.5µg/mL). The amount of individual lipids present in 2µg protein from WT spinal cord lysates was determined by HPTLC and is referred to as 1x (PI= 2 ng, GalCer= 200ng, CO= 120ng, GS= 120ng, Gb4= 24ng, SpH= 50ng, GM1= 0.6ng, Lac= 20ng, PE= 40ng). In the analysis of single lipids, 1x, 10-fold less (0.1x) or 10-fold more (10x) were used per coverslip. Gb4, PE, SpH and Lac were from Matreya LLC and CO, GalCer, GS, GM1 and PI were from Sigma-Aldrich. GalCer, GS and PI were dissolved in chloroform/methanol (2:1), and CO, PE, SpH, Gb4, GM1 and Lac in chloroform/methanol (1:2). For each lipid, the corresponding solvent was used as control. To confirm the presence of lipids following coating, lipid extraction of the coverslip was performed, and lipid content was quantified. Lipid-coating efficacy was measured by HPTLC and approximately 90% of the coated lipids were present in the coverslips. For each condition, 5,000 cells/coverslip were plated in triplicate and immunostained 12h later against βIII-tubulin (Promega, 1:2,000). In at least 100 neurons/condition, the longest neurite was traced using NeuronJ. Similar experiments were performed with cortical and hippocampal neurons isolated from E17.5 WT and shi embryos as described (Dent et al., 1999; Kaech and Banker, 2006), respectively. For cortical neurons, 50,000 cells/coverslip were maintained for 48 h and for hippocampal neurons, 16,500 cells/coverslip were maintained for 72 h.

Evaluation of toxicity. DRG neurons were plated on top of either laminin, solvent, CO or SpH. Twelve hours later, cells were washed and incubated with 1µg/mL calcein (Invitrogen) for 30 min followed by 10µg/mL propidium iodide for 5 min. Cells were then washed and 10x magnification pictures were taken. The percentage of viable cells (cells where nonfluorescent calcein AM is converted to a green-fluorescent calcein) was determined.

Rho inactivation assay. WT DRG neurons were plated onto coverslips coated with laminin, myelin, solvent, CO or SpH, as described above. Thirty minutes before plating, cells were incubated with 1 µg/mL of C3 transferase (Cytoskeleton, Inc.), a Rho inhibitor that was maintained during the entire experiment. Twelve hours later, cells were fixed and neurite outgrowth was evaluated.

ROCK activation assay. 16,500 hippocampal neurons/coverslip were plated onto coverslips coated with either solvent or SpH (10x and 100x), as described above. A pool of 12 wells/condition was used. Seventy two hours after plating, cells were lysed in 62.5mM Tris pH 6.8 containing 2% SDS, 12.5% glycerol and 5% β-mercaptoethanol. Lysates were then immunobloted against phospho-Ser19-myosin light chain (Cell Signaling, 1:1,000), the primary phosphorylation site of MLC by ROCK, and against β-actin (Sigma-Aldrich, 1:5,000).

HPβCD treatment. C57BL/6 mice (n=24) were subjected to SCI dorsal hemisection as described above. At the time of injury, osmotic minipumps (Alzet 2006) were placed subcutaneously with a tube allowing perfusion of the injury site at a rate of 0.15µl/hour with either 27µg/g/day of HPβCD in aCSF (128mM NaCl, 2.5mM KCl, 0.95mM CaCl2, 1.9mM MgCl2; n=12), or vehicle (aCSF; n=12). Besides delivery at the injury site, 4mg/g of HPβCD were injected subcutaneously twice a week. Five weeks following injury, the SCI site was collected and lipids were extracted and quantified as described above (n=6, HPβCD-treated mice; n=6, aCSF-treated mice). In n=6 HPβCD-treated mice and n=6 aCSF-treated mice, dorsal column fibers were labeled with CT-B, and axonal regeneration was assessed as previously described.

Data analysis. Data is shown as mean±SEM. Statistical significance was determined by Student's t test or Tukey's test (One-way ANOVA) for multiple comparisons.

Results

Axonal regeneration inhibitors are present in the shi spinal cord and a standard glial scar is formed upon SCI

In shi mice, the absence of MBP leads to an almost complete absence of compact myelin in the CNS, including the spinal cord (Supplementary Fig. 1*A*). Despite that several axonal regeneration inhibitors are myelin components, western blot of spinal cord extracts showed that all canonical inhibitors are present in the shi spinal cord (Fig. 1*B*). Besides a 10- and 2.5-fold decrease in the levels of MAG and OMgp, respectively, either similar (Ephrin B3 and RGMa) or increased (Nogo-A) levels of inhibitors were found (Fig. 1*B*,*C*). Immunohistochemistry against MAG and Nogo-A showed a normal distribution of the inhibitors in the shi spinal cord (Fig. 1*D*). Following complete spinal cord transection, wild-type (WT) and shi mice displayed an equivalent glial scar as evaluated by Masson trichrome staining and CSPG and GFAP immunostaining (Fig. 1*E*,*F*). Moreover, following SCI, all axonal regeneration inhibitors were present in shi spinal cords (Fig. 1*B*) being that MAG was the only for which decreased levels (40% decrease) were found. Interestingly, following injury, increased levels of the inhibitors Nogo, OMgp and EphB3 were present in the shi spinal cord (Fig. 1*B*,*G*). In summary, despite the absence of compact myelin, the shi spinal cord contains axonal regeneration inhibitors and forms a regular glial scar following SCI.

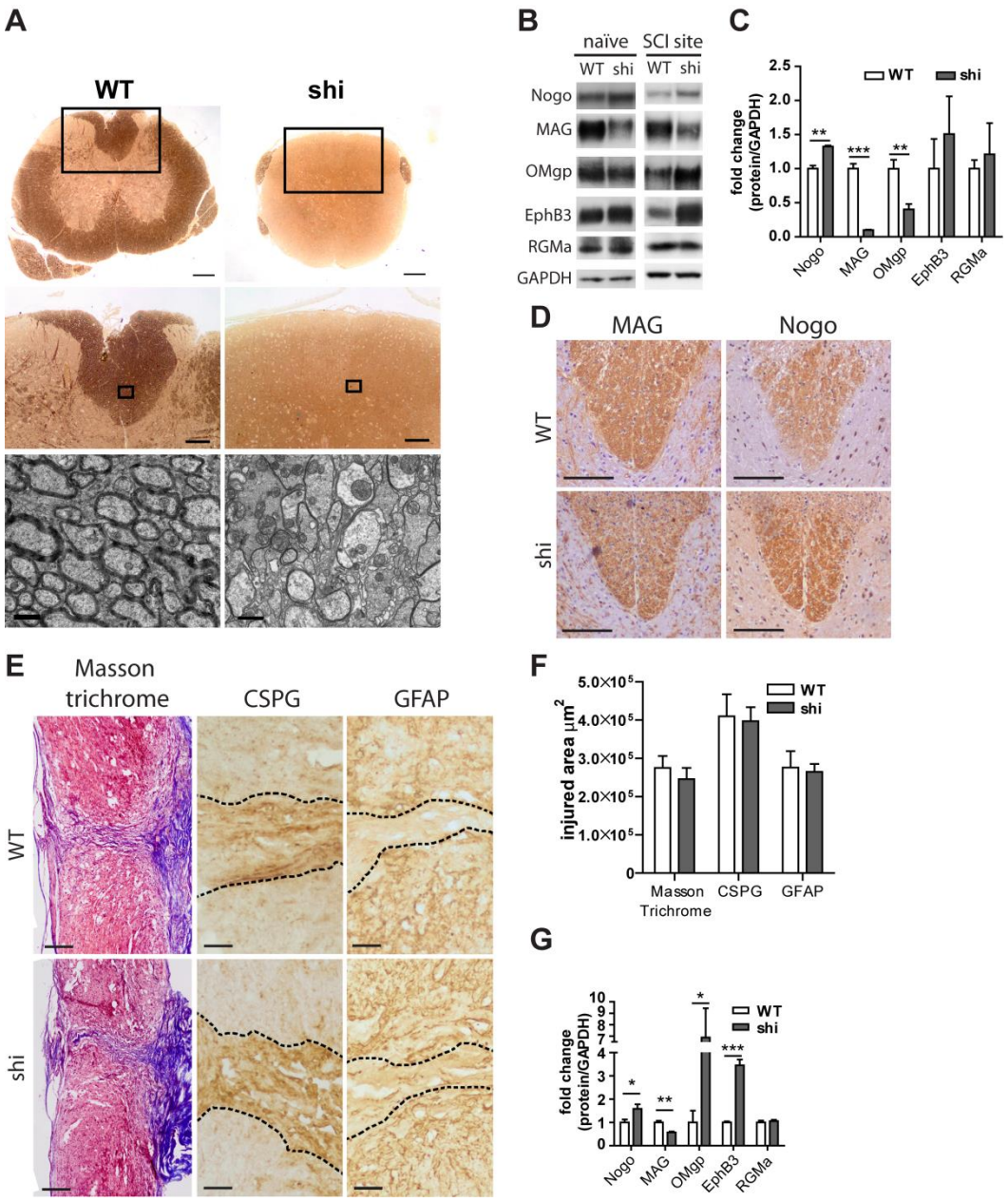

Figure 1. Shiverer mice have increased axonal regeneration and sprouting. ***A***, p-phenylene-diamine staining in thoracic spinal cords of WT and shi mice (upper panels; scale bar = 200 µm). Higher magnification of the dorsal funiculus of WT and shi spinal cord (middle panels; scale bar = 100 µm). Electron microscopy images of the white matter of WT and shi mice (lower panels; scale bar = 1 µm). ***B***, Western blot of Nogo, MAG, OMgp, EphB3, RGMa and GAPDH in spinal cord extracts of WT and shi littermates from naïve mice and mice with SCI. Representative results are shown. ***C***, Quantification of results obtained for naïve spinal cords shown in **B** (n = four mice/genotype). ***D***, Immunohistochemistry of MAG and Nogo in the dorsal funiculus of spinal cords from WT and shi littermates (scale bar = 100 µm). ***E***, Masson trichrome staining and immunostaining against CSPG and GFAP in the spinal cord of WT and shi littermates 5 weeks following complete spinal cord injury (scale bars = Masson trichrome 200 µm, GFAP and CSPG 50 µm). ***F***, Quantification of the injury area following Masson trichrome staining and immunohistochemistry against CSPG and GFAP (n = seven mice/genotype). ***G***, Quantification of the results obtained following SCI shown in **B** (n = four mice/genotype). Results represent the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001.

Axonal regeneration and sprouting are increased in shi mice

Although the shi spinal cord contains all the canonical axonal regeneration inhibitors, we asked whether the absence of compact myelin could have an impact in axonal regeneration *in vivo*. Following dorsal hemisection, and in contrast to WT axons, shi dorsal column tract axons entered the lesion site (Fig. 2*A*,*B*) and regenerated for longer distances (Fig. 2*A*,*C*). In the raphespinal tract, following complete spinal cord transection, shi 5-hydroxytryptamine (5-HT)-positive fibers found caudally to the injury site were more frequent and were capable of regenerating for longer distances (Fig. 2*D*,*E*). The ability of contralateral non-lesioned fibers to sprout in the ipsilateral injury side following lateral hemisection was used as a measure of plasticity (Lee et al., 2010). In the shi spinal cord, a 1.7-fold increase in 5-HT positive fibers was found in the ipsilateral side (WT: 33±3%; shi: 56±8%; $p<0.05$; Fig. 2*F*). The correlation with a functional improvement was not possible to evaluate given the severe shi phenotype. Of note, MAG was the only MAI decreased in the shi spinal cord following SCI (Fig. 1*G*). However, as MAG ablation is insufficient to increase axonal regeneration (Bartsch et al., 1995), decreased MAG levels are unlikely to underlie the increased axonal regeneration of shi mice. Combined, our data shows that following SCI, shi axons have increased axonal regeneration and sprouting.

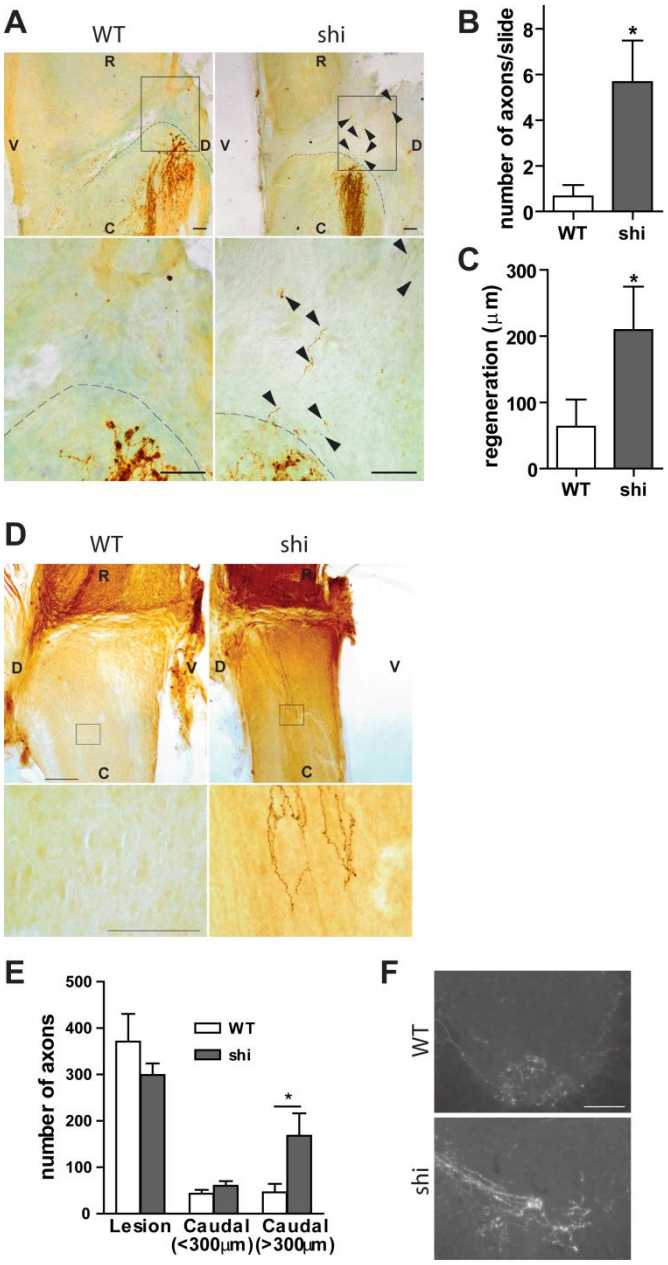

Figure 2. Shiverer mice present increased axonal regeneration. ***A***, Cholera toxin B (CT-B) immunohistochemistry in sagittal spinal cord sections of WT and shi littermates, 4 weeks following dorsal hemisection. Arrowheads highlight regenerating CT-B positive dorsal column axons in shi spinal cords. R: rostral; C: caudal; D: dorsal; V: ventral. Lower panels are higher magnifications of the selected boxed regions (scale bar = 100 μm). ***B***, Number of CT-B positive dorsal column axons able to regenerate through the glial scar in WT and shi animals (n = six mice/genotype). ***C***, Length of the longest regenerating CT-B positive dorsal column axon in WT and shi animals (n = six mice/genotype). ***D***, Serotonin (5-HT) immunohistochemistry in shi and WT spinal cords 5 weeks following complete spinal cord transection showing regenerating raphespinal fibers. R: rostral; C: caudal; D: dorsal; V: ventral. Lower panels are higher magnifications of the selected boxed regions (scale bar = 100 μm). ***E***, Quantification of regenerating raphespinal fibers (WT, n = 5; shi, n = 7). F, 5-HT immunohistochemistry of the ventral white matter of spinal cord cross sections from WT and shi littermates (WT n = 6, shi n = 7) 4 weeks following lateral hemisection to assess sprouting (scale bar = 100 μm). Results represent the mean ± SEM. *p<0.05.

Myelin lipids inhibit neurite outgrowth

Despite the alterations described in shi axons (Brady et al., 1999; Kirkpatrick et al., 2001; Andrews et al., 2006) that are generally related to decreased regeneration capacity (Hellal et al., 2011), neurite outgrowth of WT and shi dorsal root ganglia (DRG) and cortical neurons was compared and no differences were found (Fig. 3*A*). This data suggests that the intrinsic growth capacity of shi neurons does not underlie their increased regeneration *in vivo*. To evaluate whether the shi spinal cord environment might be less inhibitory than that of WT mice, and as myelin cannot be isolated from the shi CNS, we tested the effect of crude membranes prepared from spinal cords of both strains. Although DRG neurons were inhibited in the presence of both WT and shi membranes, the inhibition produced by shi membranes was lower (Fig. 3*B*), suggesting that the shi spinal cord environment presents less inhibitory cues to axonal growth. The decreased inhibitory environment of the shi spinal cord was unrelated to the lack of MBP as no effect on neurite outgrowth was produced when WT DRG neurons were plated on MBP (Fig. 3*C*). To further confirm that the decreased inhibitory environment of the shi spinal cord was unrelated to a protein component, WT DRG neurons were grown on top of protein extract from either WT or shi spinal cords. Both extracts were equally inhibitory (Fig. 3*D*).

Given that lipids account for approximately 75% of myelin dry weight, we asked whether myelin lipids would contribute to the inhibitory properties of myelin. When WT DRG neurons were grown on myelin protein or myelin lipids as substrates, a decreased neurite outgrowth was observed for both, demonstrating that myelin lipids also play a role as axonal regeneration inhibitors (Fig. 3*C*). To further demonstrate that the different lipid content of the shi spinal cord creates a more permissive environment to axonal growth, WT DRG neurons were grown on spinal cord lipids extracted from either WT or shi mice. In contrast to lipids from WT spinal cords, lipids from shi spinal cords did not inhibit neurite outgrowth (Fig. 3*E*,*F*). These data demonstrate that neurite outgrowth of WT DRG neurons can be modulated by exposure to different lipid milieus.

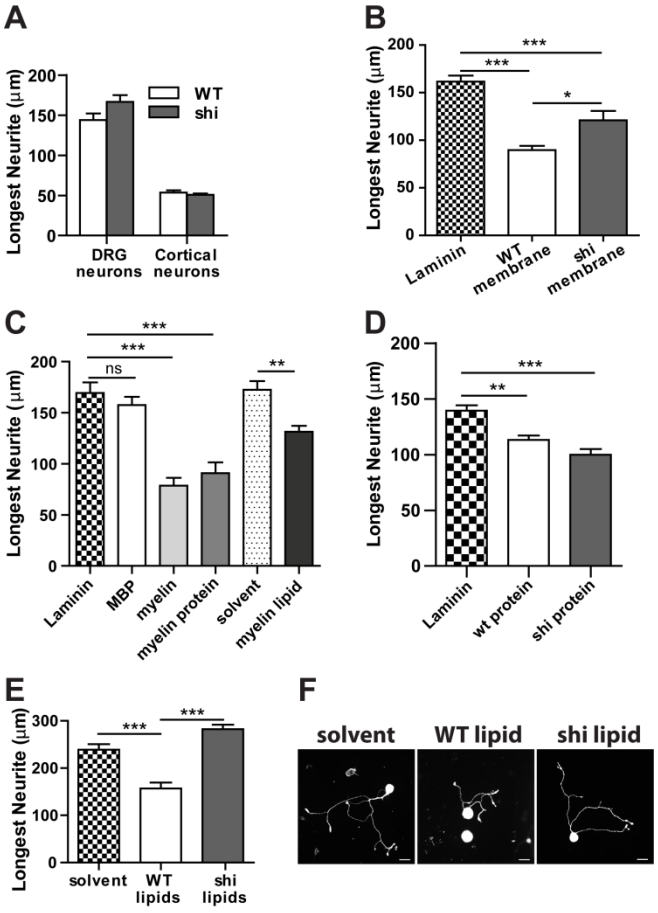

Figure 3. Myelin lipids inhibit neurite outgrowth. ***A***, Neurite outgrowth of DRG and cortical neurons from WT and shi littermates (n = 3). ***B***, Neurite outgrowth of WT DRG neurons grown on 0.1 µg of protein from WT and shi crude membranes (n = 3). ***C***, Neurite outgrowth of WT DRG neurons grown on 0.9 µg MBP, 0.5 µg WT myelin, 0.5 µg WT myelin protein or myelin lipids corresponding to 0.5 µg of WT myelin (n = 3). ***D***, Neurite outgrowth of DRG neurons grown on 3 µg of total protein from either WT or shi spinal cords (n = 3). ***E***, Neurite outgrowth of WT DRG neurons grown on lipids extracted from either WT or shi spinal cords (n=3). ***F***, Representative βIII-tubulin immunocytochemistry of **E** (scale bar 25 = µm). Results represent the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001.

Cholesterol and sphingomyelin inhibit axonal growth through a Rho-dependent mechanism

To identify the lipids that might underlie the increased axonal regeneration in the shi spinal cord, we compared the lipid composition of WT and shi spinal cords. Among others, the most abundant myelin lipids were analyzed, namely: CO, Cer, phospholipids (PE, PC, PI, PS and SpH), GM1 and GalCer (Norton and Poduslo, 1973). In the shi spinal cord we observed a decrease of most lipids (namely CO, GS, GalCer, SpH, PE, Gb4, Lac and GM1), an increase of CE and PI, and normal

amounts of Cer+PE, PS, PC and TG (Fig. 4*A*). From all the lipids with abnormal concentrations in the shi spinal cord, only CO and SpH inhibited DRG neurite outgrowth in a dose-response manner (Fig. 4*B*,*C*). This effect was unrelated to toxicity as no dead cells were found in any condition, as examined by calcein viability assay (Supplementary Fig. 4*D*). The effect of both CO and SpH was not restricted to DRG neurons as these lipids also produced a dose-dependent inhibitory effect in neurite outgrowth of hippocampal (Fig. 4*E*) and cortical neurons (Fig. 4*F*). Interestingly, when hippocampal neurons were grown on CE, that shares the same backbone structure as CO, the esterification of CO reduced its ability to inhibit neurite outgrowth (Fig. 4*E*), suggesting a specific inhibitory effect of unesterified CO.

The RhoA/ROCK pathway is the major mediator of myelin inhibition (Yiu and He, 2006), and the inhibitory effect of myelin can be reverted by the Rho inhibitor C3 transferase (Winton et al., 2002). To assess whether myelin lipids share similar pathways to block axonal regeneration than those described for myelin proteins, DRG neurons were grown on the inhibitory substrates myelin, CO and SpH and in the presence of C3 transferase. As expected, C3 was able to overcome myelin inhibition (Fig. 4*G*). Inhibition by both CO and SpH was also relieved by C3 treatment (Fig. 4*G*) suggesting that lipid inhibition of axonal growth occurs, at least in part, through a Rho-dependent mechanism. Despite that treatment with C3 improved neurite outgrowth in DRG neurons plated onto CO and SpH, given that the rescue was complete in neurons plated on SpH, we evaluated the Rho-mediated signaling cascade by measuring the levels of phosphorylated myosin light chain (p-MLC). Increased p-MLC is the downstream outcome of the activation of the effector molecule of Rho, Rho-kinase (ROCK) (Mueller et al., 2005). In the presence of SpH, p-MLC was increased (Fig. 4*H*,*I*), further supporting a lipid-based Rho-mediated inhibition.

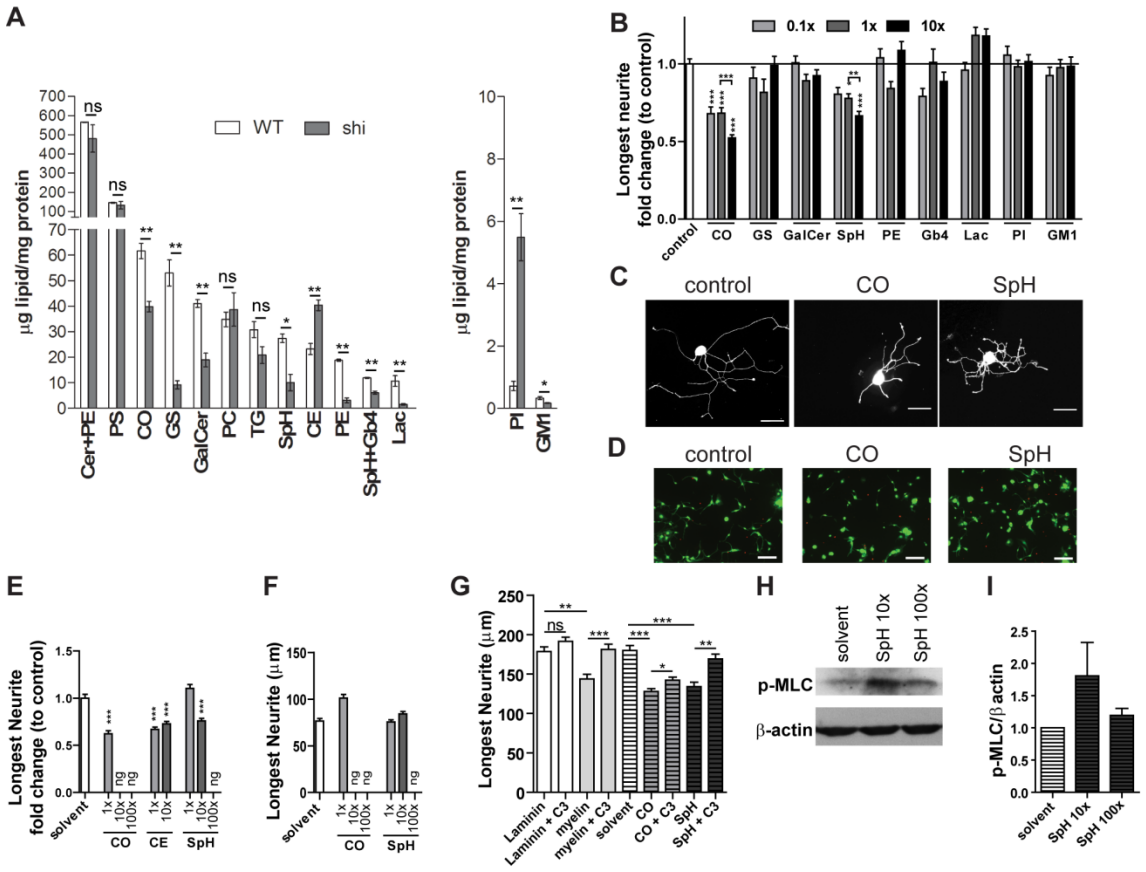

Figure 4. Cholesterol and sphingomyelin inhibit neurite outgrowth through a Rho dependent mechanism. ***A***, Quantification of lipids extracted from WT and shi spinal cords analyzed by high performance thin layer chromatography (n = three mice/genotype). ***B***, Effect of individual lipids in neurite outgrowth of DRG neurons (n = 3). ***C***, Representative βIII-tubulin immunocytochemistry of DRG neurons grown on top of solvent (control), CO and SpH coated coverslips (scale bar = 50µm). ***D***, Representative images of live/dead assay of DRG neuron cultures grown on top of solvent, CO or SpH. Green- calcein, red- Propidium iodide (scale bar = 100 µm). ***E***, Neurite outgrowth of hippocampal neurons plated on CO, CE and SpH (n = 3). ***F***, Neurite outgrowth of cortical neurons plated on CO and SpH (n = 3). ***G***, Neurite outgrowth of DRG neurons plated on top of myelin, CO or SpH in the presence or absence of the Rho inhibitor C3 transferase (n = 3). ***H***, Western blot against p-myosin light chain (p-MLC) and β-actin of hippocampal neurons grown on top of either solvent or different SpH concentrations (10x and 100x). ***I***, Quantification of **H**. Results represent the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ns: non statistical and ng: no growth detected.

Reduction of lipid levels in the spinal cord injury site through 2-hydroxypropyl-β-cyclodextrin delivery promotes axonal regeneration

To further confirm the inhibitory role of CO and SpH *in vivo*, WT mice with SCI were treated with 2-hydroxypropyl-β-cyclodextrin (HPβCD), a drug capable of reducing the levels of CO and sphingolipids, as already demonstrated in models of Niemann-Pick type C (Abi-Mosleh et al., 2009; Liu et al., 2009; Aqul et al.,

2011) and Alzheimer's disease (Yao et al., 2012). When comparing the changes in lipid content induced by SCI in WT mice, SpH and CO remained unchanged (as well as PC, Cer+PE, Gb4+SpH and GalCer), whereas CE, PE, PI and GS+PS increased at the SCI site (Fig. 5*A*). At the SCI site and following HPβCD delivery, we observed decreased levels of inhibitory lipids namely CO (28% decrease), CE (26% decrease) and SpH (21% decrease) (Fig. 5*B*) and also of others (Fig. 5*B*), although PC levels were not affected (Fig. 5*B*). Of note, in the uninjured spinal cord, administration of HPβCD did not decrease the levels of either inhibitory lipids or of the other lipids analyzed (Fig. 5*C*). In HPβCD-treated mice, improved axonal regeneration of dorsal column fibers was obtained, with a 2.5-fold increased number of axons being able to enter the lesion site (Fig. 5*D*,*E*), and a 2-fold increased length of regenerating axons (Fig. 5*D*,*F*). Combined, this study identified CO and SpH as new myelin lipids that inhibit axonal regeneration through the glial scar and determined that HPβCD may be used to reduce the levels of inhibitory lipids in the injury site and improve axonal regeneration.

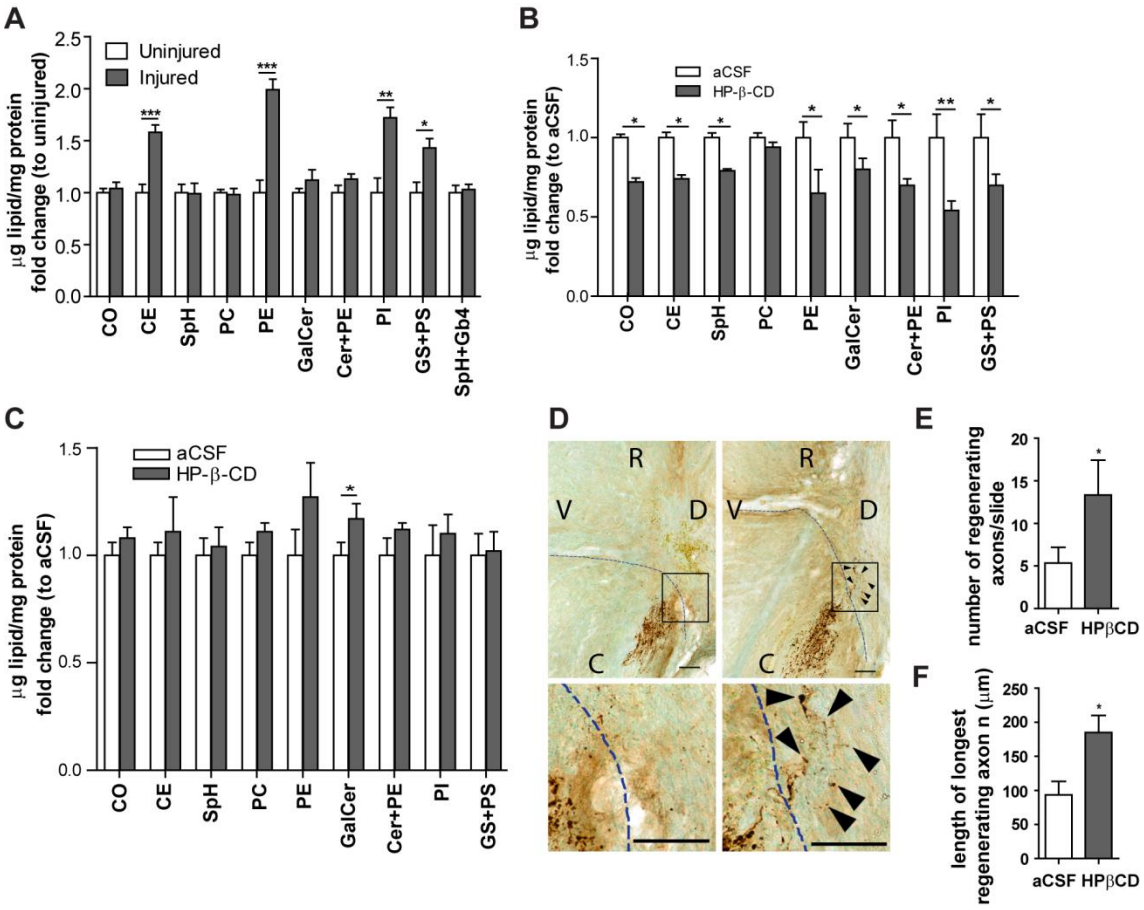

Figure 5. 2-hydroxypropyl-β-cyclodextrin delivery promotes axonal regeneration following SCI. ***A***, Quantification of CO, CE, SpH, PC, PE, GalCer, Cer, PI, GS, PS and Gb4 extracted from uninjured or injured spinal cord by high performance thin layer chromatography (n = six mice/condition). ***B***, Quantification of CO, CE, SpH, PC, PE, GalCer, Cer, PI, GS and PS in the spinal cord injury site from aCSF- and HPβCD-treated mice, 5 weeks following dorsal hemisection (n = six mice/condition). ***C***, Quantification of CO, CE, SpH, PC, PE, GalCer, Cer, PI, GS and PS in uninjured spinal cord from aCSF- and HPβCD-treated mice, 5 weeks following dorsal hemisection (n = six mice/condition). ***D***, CT-B immunohistochemistry in sagittal spinal cord sections of aCSF- and HPβCD-treated animals, 5 weeks following dorsal hemisection. Arrowheads highlight regenerating CT-B positive dorsal column axons in HPβCD-treated spinal cords. R: rostral; C: caudal; D: dorsal; V: ventral. Lower panels are higher magnifications of the selected boxed regions in the upper panels (scale bar = 100 µm). ***E***, Number of CT-B positive dorsal column axons able to regenerate through the glial scar in aCSF- and HPβCD-treated mice (n = six mice/condition). ***F***, Length of the longest regenerating CT-B positive dorsal column axon in aCSF- and HPβCD-treated mice (n = 6mice/condition). Results represent the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001.

Discussion

Our work shows that CO and SpH are myelin-associated lipid inhibitors that modulate axonal regeneration, and demonstrates that HPβCD delivery reduces the levels of lipids in the SCI site promoting axonal regrowth. Lipids represent 75% of myelin dry weight. From these, CO is the most abundant, accounting for 28% of the myelin lipid content, whereas SpH accounts for 4%. Although CO is an essential component of the plasma membrane crucial for axonal growth, CO accumulation in the mammalian brain is a risk factor for neurodegenerative diseases including Alzheimer's disease (Puglielli et al., 2003) and Niemann-Pick (Aqul et al., 2011). In the scenario of injury, with the presence of myelin debris within the injury site, myelin-derived CO is presented to regenerating axons in a different form from glial-derived CO. Whereas glial-derived CO can stimulate axon growth by providing lipoproteins as a source of both CO and apolipoprotein E to regenerating axons, free CO i.e., without the context of a lipoprotein particle, as is the case of myelin-derived CO, fails to enhance axon extension (Hayashi et al., 2004). In the case of SpH, its accumulation, which is the hallmark of Niemann-Pick disease, leads to changes in plasma membrane that similarly to CO, also culminate in neurodegeneration (Ledesma et al., 2011). As such, we propose that following CNS injury, when growing axons need to elongate through an environment filled with myelin debris, exposure to free CO and to SpH contributes to axonal growth inhibition. This mechanism may also underlie, at least in part, the increased axonal regeneration in the PNS. Despite that peripheral and central myelin have similar CO and SpH content, as dedifferentiated Schwann cells and invading macrophages are able to phagocyte and clear myelin debris following PNS injury (Stoll et al., 1989), regenerating axons are probably not exposed to a lipid-rich environment, in contrast to the CNS where such clearance is not as effective.

Accumulation of unesterified CO and SpH is the hallmark of Niemann-Pick disease, which culminates in neurodegeneration (Aqul et al., 2011; Ledesma et al., 2011). Interestingly, cultured neurons from mice lacking Niemann-Pick type C1 protein (npc1 knockout mice) displayed increased rate of growth cone collapse that was mediated by ROCK activation and reverted by ROCK inhibition (Qin et al., 2010). In npc1 mice, administration of HPβCD delays the onset of clinical symptoms by reducing the buildup of CO and sphingolipids within the nervous tissue (Davidson et al., 2009; Aqul et al., 2011). HPβCD is a well-tolerated

FDA-approved drug used in animal models and in human clinical trials, known for its ability to form inclusion complexes, remove cholesterol from membranes and increase cholesterol trafficking (Davidson et al., 2009; Aqul et al., 2011; Matsuo et al., 2013). Here we show that following HPβCD delivery, decreased levels of inhibitory lipids, namely CO, CE and SpH are specifically obtained in the SCI site whereas uninjured spinal cord segments have unaltered lipid composition. Upon spinal cord injury, HPβCD may exert a generalized sequestering effect allowing the removal of lipids from the injury site, and thus relieve the inhibitory level within the glial scar. Although HPβCD did not seem to have a clearly defined lipid specificity, we did not observe any lipid changes in the treated un-injured spinal cord, indicating that this non-toxic agent does not lead to lipid dysregulation in normal tissues. In addition, HPβCD treatment led to increased axonal regeneration, supporting that HPβCD delivery should be considered as a therapeutic option in the context of SCI.

At the molecular level, inhibition induced by CO and SpH could be reverted by the Rho inhibitor C3 transferase. In the case of SpH, for which a stronger effect was observed with C3 transferase, we further demonstrate the downstream activation of ROCK as increased phosphorylated levels of its substrate, MLC, are generated. These data supports that both myelin proteins and lipids signal inhibition through similar pathways involving Rho activation. CO and SpH may act as ligands to induce receptor-mediated activation of the Rho signaling cascade, or alternatively they can augment receptor activation leading to increased Rho activity. Whether CO and SpH engage the same receptors (e.g. Nogo receptor and PirB) as several structurally unrelated myelin proteins is to be unraveled. Specific binding sites for CO have been identified in G-protein-coupled receptors (Cherezov et al., 2007; Liu et al., 2012). As such, CO and/or SpH at the extracellular matrix in which axons are growing may interact and engage other receptors eliciting a signaling cascade that impairs axonal growth. Sulfatide has also been identified as a lipid that specifically inhibits neurite outgrowth of retinal ganglion cells through a Rho-mediated mechanism (Winzeler et al., 2011), although the pathway by which this occurs remains poorly understood. The next challenge will be to further characterize the pathways by which lipids mediate the repression of neurite outgrowth.

In summary our work shows that myelin lipids are important modulators of axonal regeneration that should be considered as critical targets in strategies

aiming at improving axonal growth following injury. Moreover, we provide the initial data supporting that the FDA-approved HPβCD delivery could be tested in the context of SCI.

Acknowledgments

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Chapter II

Differential activation and transport of injury signals contributes to the failure of a dorsal root injury to increase the intrinsic growth capacity of DRG neurons

Abbreviated title: Negative injury signals inhibit regeneration after dorsal root injury

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Abstract

Following peripheral nervous system injury, besides increased cAMP, the positive injury signals ERK, JNK and STAT-3 are locally activated and retrogradely transported to the cell body, where they induce a pro-regenerative program. Here, we used dorsal root ganglia (DRG) neurons which comprise a central branch that does not regenerate and a peripheral branch that regrows after lesion, to further understand the importance of injury signaling for successful axon regeneration. Although injury to the central branch of DRG neurons (dorsal root injury-DRI) activates the above positive injury signals and increases cAMP levels, it does not elicit the gain of intrinsic growth capacity nor the ability to overcome myelin inhibition, as occurs after injury to the peripheral branch (sciatic nerve injury-SNI). Besides, by blocking ERK activation and adenylyl cyclase activity, we show that the gain of intrinsic growth capacity of DRG neurons after injury is independent of ERK and cAMP. Antibody microarray analysis of axoplasm from rats with either DRI or SNI following dynein immunoprecipitation revealed a broad differential activation and transport of signals after each injury type and further supported that ERK, JNK, STAT-3 and cAMP signaling pathways are probably minor contributors to the differences in the intrinsic axon growth capacity observed in these injury models. From the injury signals differentially activated after DRI and SNI, we specifically identified increased levels of Hsp-40, ROCK-II and GSK3β after DRI, not only in axons but also in DRG cell bodies. In summary, our work shows that activation and transport of canonical positive injury signals is not sufficient to promote increased axonal growth capacity, and that a limited regenerative response after DRI may be accounted by the differential activation of inhibitory injury signals including ROCKII and GSK3β.

Introduction

While axon regeneration in the central nervous system (CNS) is unsuccessful, adult peripheral nervous system (PNS) axons can spontaneously regenerate to a considerable extent and are therefore generally used as a model to identify players that promote axon regrowth. Within the PNS, sensory dorsal root ganglia (DRG) neurons have been widely used in axonal regeneration studies given their peculiar nature. DRG neurons are pseudounipolar, possessing two branches: a peripheral branch that innervates sensory organs and a central branch that enters the spinal cord through the dorsal root and ascends to the brainstem, forming the dorsal column fibers (Devor, 1999). When injured, the two branches have different regenerative capacities. While an injury to the peripheral branch is followed by successful regeneration, injury to the central branch, either dorsal root injury (DRI) or spinal cord injury, fails to display a similar effect (Smith and Skene, 1997). In the case of DRI, sensory axons regenerate within the dorsal root but stop after contact with the dorsal root entry zone (DREZ; the transition zone between the spinal cord and the dorsal root), making the DRI a simplified model to study CNS regeneration. Initially, the inability of sensory axons to regenerate after DRI has been attributed to the presence of inhibitory molecules in the DREZ (Zhang et al., 2001; Beggah et al., 2005). However, recent reports suggest that the formation of new synapses when regenerating axons enter the spinal cord stabilizes them, inhibiting further growth (Di Maio et al., 2011). Besides extracellular cues, cell-intrinsic differences have been described in DRG neurons subjected to either DRI or peripheral injury. Whereas a peripheral injury triggers the expression of several regeneration associated genes (RAGs), including activating transcription factor 3 (ATF-3), growth-associated protein 43 (GAP-43) and CAP-23 among others (Schreyer and Skene, 1993; Mason et al., 2002; Seijffers et al., 2006), DRI fails to elicit a similar response. The reasons underlying the differential activation of a pro-regenerative program after SNI and DRI remain elusive.

It is widely acknowledged that injury signaling is pivotal to mount a pro-regenerative program (Mar et al., 2014). Seminal studies done in Aplysia have shown that injection of axoplasm from injured nerves into naïve neurons is accompanied by increased growth. The hypothesis raised to explain this observation was that axonal injury triggers the local activation of injury signals that are retrogradely transported to the cell body and then imported to the

nucleus (Ambron et al., 1995). Later, and further supporting this hypothesis, extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and signal transducer and activator of transcription 3 (STAT-3) have been identified as positive injury signals locally activated following peripheral injury and retrogradely transported to the cell body, playing a crucial role in mounting the response to injury (Cavalli et al., 2005; Perlson et al., 2006; Rishal and Fainzilber, 2010; Ben-Yaakov et al., 2012). Specifically, pERK is involved in the retrograde signal that initiates regeneration (Perlson et al., 2005); JNK signaling has been implicated in the reorganization of the axonal cytoskeleton and in neurite regeneration (Barnat et al., 2010) and STAT-3 contributes to the initiation of axonal regeneration (Bareyre et al., 2011) and to neuronal survival after injury (Ben-Yaakov et al., 2012). Besides injury signaling, increased levels of cAMP in DRG neurons after PNS injury have long been associated with increased axonal elongation (Cai et al., 1999; Cai et al., 2001; Neumann et al., 2002; Qiu et al., 2002; Nikulina et al., 2004). Nevertheless, recent reports have questioned the extension of cAMP effects (Blesch et al., 2012). Downstream of injury signaling, major transcriptional alterations underlie the increased regeneration ability following peripheral injury (Costigan et al., 2002). Some of the transcription factors are activated by the above injury signals: ERK activates ETS domain-containing protein (Elk-1) (Perlson et al., 2005), JNK activates c-Jun and ATF3 (Lindwall and Kanje, 2005) and the cAMP pathway activates cAMP response element-binding protein (CREB) (Gao et al., 2004). Together, these will lead to a profound change of the transcriptional profile of injured neurons contributing to their survival and regeneration through the expression of an array of RAGs including neuropeptide Y (NPY), vasoactive intestinal peptide (Vip), ATF-3, arginase 1 (Arg-1) and GAP-43, among others (Ylera et al., 2009).

Here we observed that despite DRI is unable to elicit a robust increase in axon growth, it is accompanied by the activation and retrograde transport of the canonical positive injury signals ERK, JNK and STAT-3 and increased levels of cAMP. Besides, we identified broad signaling differences between DRI and peripheral injury including increased levels of signals associated with decreased axonal growth namely, Rho-associated kinase (ROCK-II) and glycogen synthase kinase 3 β (GSK3β), that may contribute to the limited regeneration capacity after a central branch injury.

Materials and methods

Surgical procedures. Rats were handled according to European Union and National rules. All animals were maintained under a 12-h light/dark cycle and fed with regular rodent's chow and tap water ad libitum. For the animal models of injury 8-10 weeks old Wistar rats were used. For sciatic nerve injury (SNI), rats were anesthetized with ketamine (75 mg/kg)/medetomidine (0.5 mg/kg). The sciatic nerve was exposed at the mid-thigh and crush was then performed using Pean forceps, twice for 15 seconds. Analgesia (butorphanol 3 mg/kg, twice a day) was performed for 48 h following injury. For dorsal root injury (DRI), rats were anesthetized and laminectomy at lumbar vertebrae 2 was performed exposing the dorsal roots of DRG L4,5. The L4,5 dorsal roots were then crushed using fine forceps, twice for 15 seconds. Analgesia was performed as described above.

Quantitative PCR. One week after either SNI or DRI, rats were sacrificed with an overdose of ketamine/medetomidine and L4,5 DRGs were collected; naïve rats were used as control (n=4 rats per group were used). RNA was extracted from DRGs (Qiagen), and cDNA was synthesized by reverse transcription (Invitrogen) using 1 µg of RNA. qRT PCR was then performed using iQ supermix (Bio-Rad) and primers designed using Beacon designer: NPY (sense: 5'-GCTCGTGTGTTTGGGCATTCTG-3'; antisense: 5'-GTGTCTCAGGGCTGGATCTCTTG-3'), Vip (sense: 5'-GTCACTCATTGGCAAACGAATCAG-3'; antisense: 5'-CTCCCTCACTGCTCCTCTTCC-3'), GAP-43 (sense: 5'-GATAACTCGCCGTCCTCCAAG-3'; antisense: 5'-CTACAGCTTCTTTCTCCTCCTCAG-3'); Arg-1 (sense: 5'-GACATCAACACTCCGCTGACAAC-3'; antisense: 5'-CCAGGGTCCACATCTCGCAAG-3'); ATF-3 (sense: 5'-TCTGGAGATGTCAGTCACCAAGTC-3'; antisense: 5'-CCTTCAGTTCGGCATTCACACTC-3'); Hypoxanthine-guanine phosphoribosyltransferase (Hprt) (sense: 5'-ATGGACTGATTATGGACAGGACTG-3'; antisense: 5'-GCAGGTCAGCAAAGAACTTATAGC-3').

Neurite outgrowth assays. One week after either SNI or DRI, animals were sacrificed with an overdose of ketamine/medetomidine and L4,5 DRGs were collected. DRG neuron cultures were performed as described (Miranda et al., 2011). Briefly, DRG were collected and digested with 0.125% collagenase IV-S (Sigma-Aldrich) for 3 h at 37°C. A single-cell suspension was obtained by trituration with a fire-polished Pasteur pipette and centrifuged over a 15%

albumin cushion for 10 minutes at 200g. Cells were then plated in poly-L-lysine (20 µg/mL)/laminin (5 µg/mL; Sigma-Aldrich) or in poly-L-lysine (20 µg/mL)/myelin-coated (2.4 µg/coverslip; myelin was isolated from the spinal cord of 16 weeks-old WT mice as detailed (Norton and Poduslo, 1973)) 13 mm coverslips and maintained at 37 ˚C for 12 h. Subsequently, cells were fixed, immunostained against βIII tubulin (1:2000, Promega) and traced with NeuronJ (an ImageJ plugin). The length of the longest neurite was measured in at least 100 neurons per condition.

Axoplasm extraction. Immediately after SNI or DRI, a knot was performed with 4/0 suture thread proximally to the DRG to restrain the axonal transport of injury signals (Fig. 2*A*). Eight or 24 h after each injury type, rats were sacrificed and nerves were collected to 100 µl of PBS containing protease inhibitors (GE Healthcare) and 1 mM ortovanadate (Sigma-Aldrich). Nerves from naïve rats were used as control. Axoplasm was collected by gently squeezing the nerves with a pestle followed by a centrifugation at 15,000 g at 4 ºC for 10 minutes, as described (Hanz et al., 2003). This cycle was repeated 2 more times. After the last centrifugation, supernatant was collected and 25 µg of each protein sample were run in 12% SDS-PAGE.

DRG protein extract. L4,5 DRG of animals with either SNI or DRI were collected 17-20 h after lesion, respectively; naïve animals were used as a control (n=4/group). The tissue was then homogenized in PBS containing protease inhibitors (GE Healthcare), 1mM ortovanadate and 0.3% Triton X-100, sonicated and centrifuged for 10 minutes at 15,000 g at 4 ˚C. The supernatant was collected and 25 µg of each protein sample were run in 12% SDS-PAGE.

Dynein immunoprecipitation. Dynein immunoprecipitation was performed as described elsewhere (Perlson et al., 2005). Briefly, 500 µg of axoplasm protein was precleared for 1 h at 4 ˚C with protein G magnetic beads (GE Healthcare) and then incubated with 10 µg of anti-dynein antibody (Millipore) overnight at 4 ˚C. The immune complex formed was collected by incubation for 2 h at room temperature with protein G magnetic beads (GE Healthcare), and then boiled in sample buffer (31.25 mM Tris-HCL pH 6.8; 1 % SDS; 12.5 % glycerol; 0.02 % bromophenol blue; 1.25 % β-mercaptoethanol). The immunoprecipitated samples were then run in 12% SDS-PAGE.

Immunoblots. Twenty five µg of either axoplasm, DRG extract or dynein immunoprecipate were immunobloted against pERK (T202/Y204) and total-ERK (both 1:2,000, Cell Signaling), pJNK ((T183/Y185) and total-JNK (both 1:1,000, Cell Signaling), pSTAT-3 (Y705) and total-STAT-3 (both 1:2,000, Cell Signaling), β-actin (1:5,000, Sigma-Aldrich), GSK3α/β (1:2,000, Santa-Cruz Biotechnologies), C-terminal Src kinase (Csk) (1:1,000, Santa-Cruz Biotechnologies), proto-oncogenic cytoplasmic tyrosine kinase (Src) (1:1,000, Signalway Antibody), Hsp-40 (1:1,000, Santa-Cruz Biotechnologies), mitogen-activated protein kinase kinase (MEK6) (1:1,000, Enzo Life Sciences), Caspase 4 (1:1,000, Enzo Life Sciences), Polo-like kinase 3 (Plk3) (1:500, Abgent), ROCK-II (1:1,000, Millipore), total-collapsin response mediator protein-2 (CRMP-2), pCRMP-2 (both 1:1,000, Kinasource) and GAPDH (1:1,000, Santa-Cruz Biotechnologies). Quantitative analysis of Western blots was performed using PDQuest (Bio-rad).

In vitro conditioning assays. DRG from 8 weeks-old Wistar rats were collected and DRG cultures were performed as described above. Cells were either fixed 12 h after plating (naïve type of growth), or grown for 24 h and treated with trypsin-EDTA (Invitrogen) for 5 min, replated and allowed to grow for 12 h (conditioned type of growth). The *in vitro* assay was performed in the presence of the ERK inhibitor U0126 (Calbiochem; 10 µM in 0.1 % DMSO), or of the adenylyl cyclase inhibitor 9-cyclopentyladenine monomethanesulfonate (9-CPA; 100 µM in H2O, Sigma-Aldrich). During the assay, cells were exposed to the drug immediately following tissue collection and the drug was present in all culture mediums. Vehicle was used as control for both drugs. ERK inhibition was confirmed by Western blot against pERK. The efficiency of 9-CPA was assessed by measuring the levels of cAMP, as described below.

cAMP quantification and immunohistochemistry. L4,5 DRG of rats with either SNI or DRI were collected 1 day after lesion; naïve animals were used as control (n=4 per group). Tissues were immediately collected to liquid nitrogen, homogenized in ice cold 6 % trichloroacetic acid solution and centrifuged for 15 minutes at 2,000 g at 4 ˚C. The supernatant was washed 4 times with 1 ml of water saturated diethyl ether. The aqueous phase was then dried under a stream of Nitrogen. cAMP levels were measured by an enzyme immune assay according to the manufacturer's instructions (GE Healthcare). For cAMP immunohistochemistry, L4,5 DRG of animals with either SNI or DRI were collected 1 day after lesion and were formalin fixed, processed and paraffin embedded; naïve animals were used

as control (n=4 per group). Paraffin sections were cleared, hydrated and incubated in blocking buffer (10 % fetal bovine serum, with 0.3 %Triton X-100) for 1 h at room temperature and then incubated with anti-cAMP primary antibody diluted in blocking buffer (1:500 Millipore) overnight at 4 ºC, followed by incubation with biotinylated secondary antibody in blocking buffer (1:200, Vector laboratories) and avidin peroxidase (Elite ABC kit, Vector laboratories). Antigen visualization was performed using DAB (Vector laboratories). Sections were then dehydrated, mounted in DPX and pictures were taken at 10 x magnification (Olympus).

Antibody microarray analysis. Axoplasm of L4,5 ligated nerves from rats with either SNI (a pool of 18 animals) or DRI (a pool of 6 animals) was collected 8 h after injury. Dynein immunoprecipitation was performed as described above. The immune complexes formed were dissociated by two incubations of 10 minutes with 0.1 M glycine pH 2.5, at room temperature. The solution was neutralized with 1 M Tris pH 9 and then incubated with protein G magnetic beads (GE Healthcare) to remove the IgGs. The collected supernatant (immunoprecipitation product without IgGs) was analyzed using the Kinex™ Antibody Microarray (Kinexus). The Kinex™ Antibody Microarray tracks both protein expression (with ~510 pan-specific antibodies) and phosphorylation (with ~340 phospho-site-specific antibodies) using highly validated commercial antibodies. Briefly, 50 µg of lysate protein from each sample (axoplasm following dynein immunoprecipitation from animals with either DRI or SNI) was covalently labeled with a Kinexus proprietary fluorescent dye and loaded side by side on the same chip (each microarray consists of 2 identical fields). Signal quantification was performed with *ImaGene 8.0* from BioDiscovery (El Segundo, CA). Z scores were calculated by subtracting the overall average intensity of all spots within a sample from the raw intensity for each spot, and dividing it by the standard deviations (SD) of all of the measured intensities within each sample. Z ratios were further calculated by taking the difference between the averages of the observed protein Z scores and dividing by the SD of all of the differences for that particular comparison. A short list of 46 candidates was selected based on the following parameters: Z-ratio = >+/- 1.0, Error Range for adjacent duplicate spots of the same protein <30%, Globally Normalized Median Value >254, Flags (indication of the quality of the spot based on its morphology and background) = 0.0. To validate the microarray analysis, additional samples of axoplasm of ligated nerves

from rats with either SNI (a pool of 14 animals) or DRI (a pool of 7 animals) was collected 8h after injury and validation was done by Western blot- Kinetworks™.

DRG immunohistochemistry. L4,5 DRG of animals with either SNI or DRI were collected 17-20 h after lesion, formalin fixed and processed for paraffin blocks; naïve animals were used as controls (n=4/group). Paraffin sections were cleared and hydrated, antigen retrieval was performed, blocking was done with 5 % fetal bovine serum for 1 h at room temperature and the following primary antibodies were incubated overnight at 4ºC: pSTAT-3 (1:200, Cell Signaling), GSK3β (1:100, Cell Signaling), Csk (1:50, Santa-Cruz Biotechnologies), Src (1:200, Signalway Antibody), Hsp-40 (1:50, 10 mM sodium citrate, Santa-Cruz Biotechnologies), MEK6 (1:200, Enzo Life Sciences), Caspase 4 (1:100, Thermo Scientific) and ROCK-II (1:100, Millipore). Sections were then incubated with anti-rabbit IgG-alexa488 (1:1,000, Invitrogen) and mounted with vectashield (Vector laboratories). Images were taken in a fluorescence microscope (Zeiss Axio Imager) with a 10 x objective. Quantification was performed by determining the percentage of DRG neurons with positive staining.

Lentivirus production and neurite outgrowth analysis. HEK293T cells were transfected with packaging plasmids pPAX and pLP/VSVG (Dr. Relvas, IBMC) and a lentiviral vector (pLKO) coding for puromycin resistance and expressing a scrambled shRNA (Sarbassov et al., 2005) or a shRNA for Hsp-40 (Sigma-Aldrich). Forty eight hours later, viral titration was done by infecting HEK 293T cells. DRG neuron cultures from 8-weeks-old rats were performed as described above. One day after plating, cells were infected with lentivirus (5,000 IU/well) for 12-16 h, and 24 h later treated with puromycin (5μg/mL) for 48 h. For neurite outgrowth, cells were trypsinized, replated for 12 h and immunocytochemistry against βIII-tubulin (1:2,000, Promega) was conducted. At least 60 neurons/condition were traced using NeuronJ.

Statistical analysis. All values are presented as mean ± SEM. Student's t-test or 1-way-anova with Tukey's test was used.

Results

A dorsal root injury elicits the activation and retrograde transport of positive injury signals to the soma

After DRI in adult animals, sensory axons regenerate within the dorsal root but stop after contact with the spinal cord. Besides the inhibitory extrinsic environment, DRI fails to activate an intrinsic regenerative program (Seijffers et al., 2006). In accordance with previous findings (Seijffers et al., 2006), DRG neurons plated on the permissive substrate laminin collected from rats with a conditioning SNI had a robust 1.6-fold increased neurite outgrowth, whereas after DRI a marginal 1.1-fold increase was observed (Fig. 1*A*,*B*). Moreover, while DRG collected form rats primed with a SNI were able to overcome myelin inhibition, DRI was unable to generate a similar effect (Fig. 1*A*,*C*). The failure of DRI in increasing the growth capacity of DRG neurons and of overcoming myelin inhibition correlated with a decreased capacity in inducing the expression of RAGs, namely NPY, Vip, GAP-43, Arg-1 and ATF-3 (Fig. 1*D*). In summary, besides the extrinsic inhibitory milieu, axonal regeneration failure after DRI might also result from its inability to prime DRG neurons to increase their intrinsic growth capacity.

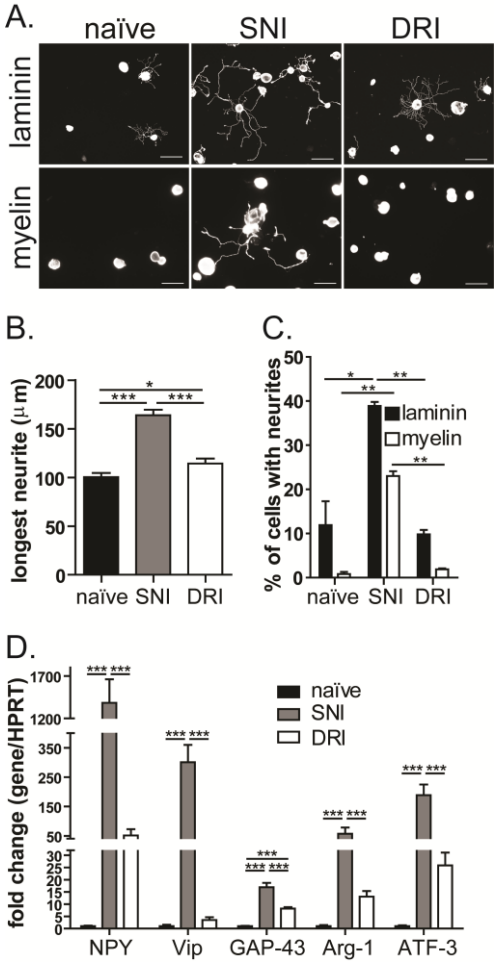

Figure 1. DRI does not elicit a robust increase in the intrinsic growth capacity of DRG neurons. ***A***, Representative βIII-tubulin immunocytochemistry of L4,5 DRG neurons from naïve rats or rats with either SNI or DRI, grown on top of laminin or myelin; scale bar 100μm. ***B***, Neurite outgrowth of DRG neurons grown on laminin. ***C***, Quantification of the percentage of cells with neurites in cultures represented in **A**. ***D***, qPCR of L4,5 DRGs from naïve rats, and from rats with either SNI or DRI (n=4/group). Results represent the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001.

Locally activated injury signals have been proposed as important contributors to the robust regenerative response after peripheral lesion (Hanz et al., 2003; Cavalli et al., 2005; Ben-Yaakov et al., 2012). To test whether the absence of a strong regenerative response following DRI could be due to failure in the activation of positive injury signals, these were tested following lesion. To do so, we used the crush-ligation paradigm where proximally to the cell body a nerve ligation is performed to trap proteins retrogradely transported from the injury site (Fig. 2*A*). Axoplasm was extracted 8 and 24h following injury and analyzed for the presence of injury signals. ERK activation was evaluated following dynein immunoprecipitation in the axoplasm. Eight h after lesion, both DRI and SNI lead to an increase in pERK bound to dynein (6-fold and 3-fold,

respectively) (Fig. 2*B*,*C*). Besides pERK, pJNK (Fig. 2*B*,*D*) and pSTAT-3 (Fig. 2*B*,*E*) were activated in the axoplasm after DRI to a similar extent as after SNI. These signals were not only activated by DRI but were also retrogradely transported and able to reach the cell body. In DRG extracts, following either DRI or SNI, increased pERK (20- and 10-fold, respectively- Fig. 2*F*,*G*) and pSTAT-3 (2.1- and 2.8-fold, respectively- Fig. 2*H*,*I*) were observed. Similar levels of pJNK in the DRG were also observed following SNI and DRI. Furthermore, immunohistochemistry of DRG neurons showed that pSTAT-3 is translocated to the nucleus following both injury types (Fig. 2*J*,*K*). In summary DRI is able to induce to a similar extent as SNI, the activation and retrograde transport of injury signals to the DRG cell body.

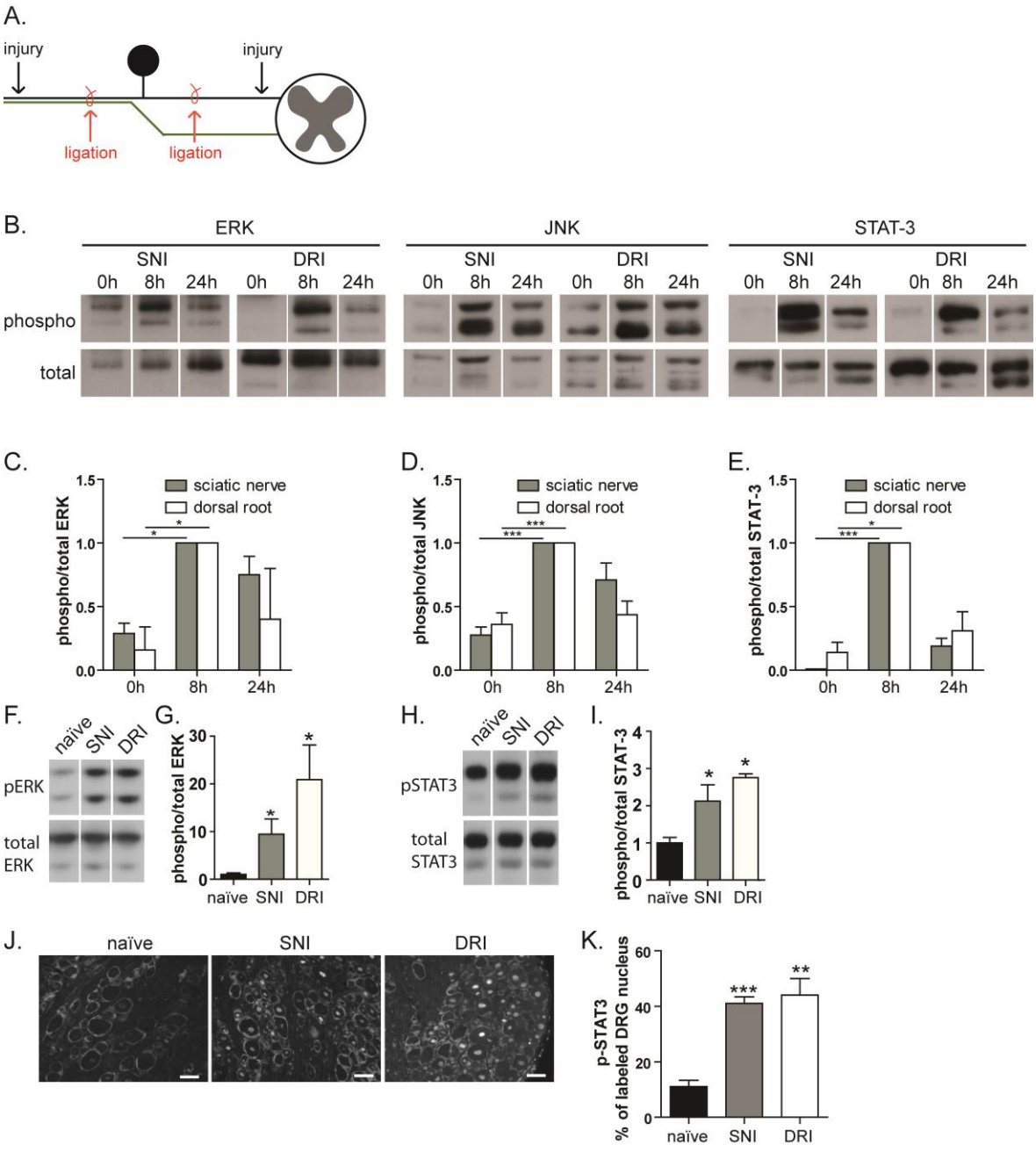

Figure 2. DRI activates positive injury signals that are retrogradely transported to the cell body. ***A***, Schematic representation of the injury-ligation model of the rat sciatic nerve and of L4,5 dorsal roots. ***B***, Representative Western blots of axoplasm collected at different time points following either SNI or DRI against pERK and total ERK (after dynein immunoprecipitation); pJNK and total JNK; pSTAT-3 and total STAT-3. ***C***, Quantification of ERK in **B** (n=3/group). ***D***, Quantification of JNK in **B** (n=6/group). ***E***, Quantification of STAT-3 in **B** (n=6/group). Representative Western blots (and respective quantifications) against ***F***,***G***, pERK, and total ERK, ***H***,***I***, pSTAT-3 and total STAT-3 in L4,5 DRGs from naïve rats and from rats with either SNI or DRI; (n=4/group). ***J***, Immunohistochemistry against pSTAT-3 of L4,5 DRG of either naïve rats or rat with either SNI or DRI. ***K***, Quantification of the percentage of neurons with labeled nucleus in **J** (n=8/group). Results represent the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001.

ERK activation is not essential for DRG neurons to trigger a regenerative response

The fact that positive injury signals are activated by DRI in the absence of an increase in the intrinsic axonal growth questions their relevance as crucial injury signals sufficient to trigger a regenerative response. Preventing ERK activation with the MEK inhibitor U0126 has been shown to inhibit axonal regeneration following peripheral nerve injury (Perlson et al., 2005). To further clarify the role of ERK in the gain of axonal growth capacity, we performed an *in vitro* assay mimicking a peripheral injury by trypsin treatment and replating naïve DRG neurons after 24 h in culture (Saijilafu et al., 2013), grown with or without U0126. The efficiency of the inhibitor was confirmed by immunoblot against pERK in cultured DRG neurons (Fig. 3*A*). Trypsin treatment increased neurite outgrowth (Fig. 3*B*), similarly to the *in vivo* priming of DRG neurons after peripheral injury. Although U0126 lead to a small decrease in neurite outgrowth of naïve DRG neurons (Fig. 3*B*), trypsin-treated DRG neurons had similar increase in neurite outgrowth with or without U0126 treatment (Fig. 3*B*), suggesting that priming DRG neurons triggers ERK-independent mechanisms that can override its absence.

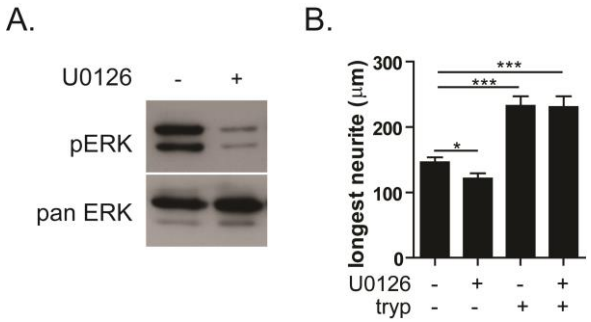

Figure 3. ERK activation is not essential for DRG neurons to trigger a regenerative response. ***A***, Western blot against pERK and total ERK in DRG neuron cultures either untreated or treated with the MEK inhibitor U0126. ***B***, Neurite outgrowth of DRG neurons treated with U0126 before or after trypsin treatment (n=3/group). Results represent the mean ± SEM. *p<0.05 and ***p<0.001.

cAMP increases after DRI and is not central for the gain of axonal growth capacity of conditioned DRG neurons

High levels of cAMP following peripheral injury have been described as essential to increase axonal regeneration (Qiu et al., 2002). Given the importance of cAMP in axon growth, we next asked if a DRI could increase the cAMP levels of DRG

neurons. Both after DRI and SNI, a 2-fold increase in cAMP levels was present in DRG neurons (Fig. 4*A*). Immunohistochemistry against cAMP of DRG following SNI or DRI also confirmed this increase (Fig. 4*B*). We went on to further evaluate the relevance of cAMP in the gain of regenerative capacity of trypsin-conditioned DRG neurons. To block cAMP synthesis, the adenylyl cyclase inhibitor, 9-cyclopentyladenine (9CPA) was used. Culturing DRG neurons with 9CPA decreased cAMP levels by more than 50%, confirming the inhibition of adenylyl cyclase (data not shown). In DRG neurons conditioned by trypsin, 9CPA treatment did not prevent the gain of neurite outgrowth capacity (Fig. 4*C*). Overall these data suggest that cAMP increase is not sufficient to promote a gain in the intrinsic growth capacity of DRG neurons and that this increased capacity can be achieved in the absence of a rise in cAMP.

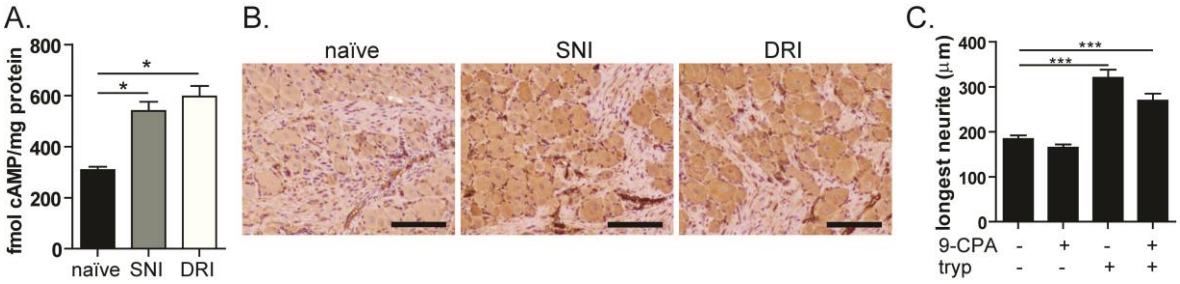

Figure 4. cAMP is not essential for DRG neurons to trigger a regenerative response. ***A***, Quantification of cAMP levels in L4,5 DRG of naïve rats or rats with either SNI or DRI (n=4/group). ***B***, Immunohistochemistry against cAMP of L4,5 DRG of naïve rats or rats with either SNI or DRI (n=4/group). ***C***, Neurite outgrowth of DRG neurons treated with the adenylyl cyclase inhibitor 9-CPA before or trypsin treatment (n=3/group). Results represent the mean ± SEM. *p<0.05 and ***p<0.001.

DRI and SNI lead to the differential activation and transport of signals in the axoplasm

Since no major variation was found in known injury signals when comparing SNI and DRI, we performed an antibody microarray to identify possible differences that could explain the fact that a SNI elicits a gain in the intrinsic growth capacity whereas DRI fails to do so. For that, using a ligation proximal to the DRG, we collected the axoplasm of dorsal roots or sciatic nerves following DRI or SNI, respectively. Following dynein immunoprecipitation the samples were analyzed using Kinex™ Antibody Microarray (Kinexus) that tracks both protein expression (with ~510 pan-specific antibodies) and phosphorylation (with ~340 phospho-site-

specific antibodies). The array analysis revealed that 28 protein targets had a significantly upregulated signal whereas 16 were significantly downregulated after DRI (Table 1).

Table 1: Target proteins for which a significant variation was found by microarray analysis of dynein-immunoprecipitated axoplasm from DRI and SNI samples. Grey- targets for which additional analysis was performed by Western blot of axoplasm; Red- targets for which Western blot results validated the microarray data.

Full Target Protein Name	Phospho Site (Human)	Microarray Z-ratio (DRI/SNI)	Western blot fold change (DRI/SNI)
Acetylated Lysine	Pan-specific	1,28	
B-cell lymphoma protein 2 alpha (Bcl2)	Pan-specific	2,14	not detected
Breast cancer type 1 susceptibility protein (BRCA1)	S1497	1,29	
Caspase 4	Pan-specific	-1,2	0,7
Caspase 5	Pan-specific	-1,26	2,2
Caveolin 1	Y14	-1,32	not detected
Cell division cycle 2-like protein-serine kinase 5 (CDC2L5, CHED)	Pan-specific	-1,21	not detected
C-terminus of Src tyrosine kinase (Csk)	Pan-specific	1,05	1,9
Cyclin-dependent protein-serine kinase 5 (CDK5)	Pan-specific	-1,07	1,7
Cyclin-dependent protein-serine kinase 8 (CDK8)	Pan-specific	4,35	
DnaJ homolog, subfamily B member 1(Hsp40)	Pan-specific	1,4	1,4
Epidermal growth factor receptor-tyrosine kinase (EGFR)	Y1110	1,79	not detected
Extracellular regulated protein-serine kinase 2 (Erk1 + Erk2)	Pan-specific	1,32	
Glycogen synthase-serine kinase 3 beta (GSK3b)	Pan-specific	1,04	3,2
Heat shock 27 kDa protein beta 1 (Hsp27)	S78	2,12	0,9
Heat shock 60 kDa protein 1 (chaperonin, CPN60)	Pan-specific	1,37	
Histone H2A variant X	S140	-1,22	
Histone H2B	S15	-1,14	
Integrin-linked protein-serine kinase 1 (ILK1)	Pan-specific	-1,21	not detected
Jun N-terminus protein-serine kinase (JNK1/2/3)	Pan-specific	-1,46	
Kit/Steel factor receptor-tyrosine kinase (Kit)	Y936	1,7	not detected
LIM domain kinase 1 (LIMK1)	Pan-specific	1,86	not detected
LIM domain kinase 2 (LIMK1 + LIMK2)	Y504+T505	1,19	not detected
MAPK/ERK protein-serine kinase 1 (MKK1, MEK1, MAP2K1)	Pan-specific	1,09	
MAPK/ERK protein-serine kinase 2 (MKK2, MEK2, MAP2K2)	T394	1,14	not detected
MAPK/ERK protein-serine kinase 6 (MKK6, MEK6, MAP2K6)	Pan-specific	1,11	3,0
Mitogen-activated protein-serine kinase p38 alpha (p38a MAPK)	Pan-specific	1,21	
Mitogen-activated protein-serine kinase p38 gamma (MAPK12, p38g MAPK, Erk6)	Pan-specific	1,03	
NF-kappa-B p65 nuclear transcription factor (NFkappaB p65)	S276	-1,03	not detected
nucleophosmin, numatrin, nucleolar protein NO38 (B23)	T234/T237	1,68	not detected
Polo-like protein kinase 3 (Plk3)	Pan-specific	1,36	2,2

Protein-serine kinase B beta (PKBb; Akt2)	Pan-specific	2,77	
Protein-serine kinase C beta 1/2 (PKCb1/2)	T500	1,2	not detected
Protein-serine kinase C beta 2 (PKCb2)	Pan-specific	1,2	0,5
Protein-serine kinase C eta (PKCh)	T655	-5,22	not detected
Protein-serine kinase C lambda/iota (PKCl/i)	T564	1,1	1,0
Protein-serine kinase C theta (PKCq)	Pan-specific	1,32	not detected
Protein-serine phosphatase 2A - alpha and beta isoforms (PP2A/Aa/b)	Pan-specific	-1,12	1,9
Retinoblastoma-associated protein 1	S807	-2,12	not detected
Retinoblastoma-associated protein 1	T356	-1,51	not detected
RhoA protein-serine kinase alpha (ROCK2)	Pan-specific	1,18	1,3
Signal transducer and activator of transcription 3 (STAT3)	S727	1,25	
Sphingosine kinase 2 (SPHK2)	Pan-specific	-1,12	not detected
Src proto-oncogene-encoded protein-tyrosine kinase (Src)	Pan-specific	-1,18	0,6

According to our previous observations related to ERK signaling, by microarray analysis, no differences were found between DRI and SNI in active ERK, nor in vimentin and Elk-1 (involved in ERK transport (Perlson et al., 2005) and signaling pathway) (Table 2). Of note, whereas a pan-specific antibody against ERK was increased after DRI (Table 1), four other pan-specific ERK antibodies did not reveal differences between DRI and SNI (Table 2), further supporting the absence of changes in ERK signaling between the two lesion types. Similar findings were obtained for JNK, as no differences between DRI and SNI were found in the active form of the protein, nor in its downstream effector c-jun (Table 2). Besides, confirming the absence of differences in STAT-3 signaling, including JAK activation, no differences were observed in phospho-Y705 pSTAT-3 in the antibody microarray (Table 2), similarly to our previous Western blot analysis, despite that phospho-S737 pSTAT-3 (considered a secondary event after Y705 phosphorylation), was upregulated after DRI (Table 1). Besides, CREB, the downstream effector of cAMP was also unchanged when comparing DRI and SNI (Table 2). In the adult CNS, intrinsic inhibitors of axon regrowth have been recently identified, including phosphatase and tensin homolog (PTEN). PTEN antagonizes the action of PI3K, leading to inactivation protein kinase B (AKT) and of mammalian target of rapamycin (mTOR) signaling (Christie et al., 2010). Of note, the PTEN/mTOR signaling was unaltered when comparing DRI and SNI (Table 2), supporting that this specific pathway is not responsible for the differences in regeneration observed after each of the lesion types.

Table 2: Selected target proteins with no significant differences in dynein-immunoprecipitated axoplasm from DRI and SNI samples as detected by microarray analysis. Number after target protein name indicates number of independent antibodies present in the microarray against that target, for which no significant variation in SNI and DRI samples was found.

Target Protein Name	Phospho Site (Human)
CREB1 (1)	S129+S133
CREB1 (2)	S133
Elk-1(1)	Pan-specific
Elk-1(1)	S383
Erk1 + Erk2 (5)	Pan-specific
Erk1 + Erk2 (1)	T185+Y187
Erk1 + Erk2 (2)	T202+Y204
JAK1 (2)	Pan-specific
JAK1 (1)	Y1034
JAK2 (1)	Pan-specific
JAK2 (2)	Y1007+Y1008
JAK3 (1)	Pan-specific
JNK1 (1)	Pan-specific
JNK1/2/3 (2)	Pan-specific
JNK1/2/3 (3)	T183 + Y185
JNK2 (1)	Pan-specific
JNK2/3 (2)	Pan-specific
JNK3 (1)	Pan-specific
Jun (1)	Pan-specific
Jun (1)	S63
Jun (2)	S73
mTOR (1)	S2448
PTEN (2)	Pan-specific
PTEN (1)	S380+T382+S385
STAT2 (1)	Y690
STAT3 (2)	Pan-specific
STAT3 (2)	Y705
Vimentin (1)	S34

In summary, the antibody microarray analysis substantiates that DRI and SNI lead to a broad differential activation and transport of signals in the axoplasm, and that the ERK, JNK, STAT-3, cAMP and PTEN/mTOR signaling pathways are probably minor contributors to the changes in the intrinsic axon growth capacity observed in these injury models.

The negative injury signals ROCK-II and GSK3β are upregulated in the DRG cell body following DRI

From the microarray shortlist, most of the candidates were chosen for further validation by immunoblot of total axoplasm collected from additional rats with either SNI or DRI (Table 1, highlighted in grey). Given the extensive analysis already described, ERK, JNK and STAT-3 were excluded from further validation. From the selected candidates, 17 were not detected by Western blot of axoplasm samples given its lower sensitivity when compared to antibody microarray analysis. From the remaining 14 candidates, 8 were detected with similar differential levels in total axoplasm as those observed by antibody microarray analysis of dynein-immunoprecipitated axoplasm: Hsp-40, Plk3, ROCK-II, MEK6, Csk and GSK3β- increased after DRI; Src and Caspase 4- decreased after SNI (Table 1). Although we excluded from subsequent analysis the 6 candidates where no consistent results were found, one should note that whereas microarray analysis was performed in dynein-immunoprecipitated axoplasm i.e., allowing the identification of proteins bound to the transport machinery, Western blot was done on total axoplasm samples, which may underlie the differences observed. From the above 8 candidates with consistent changes detected both by microarray analysis and Western blot, clear alterations in the DRG were found by immunoblot against Hsp-40 and GSK3β where a 2.0- and 1.3- fold increase following DRI respectively, was observed (Fig. 5*A,B*). Besides, by immunostaining of DRG, following DRI, a 1.4-, 1.6- and 2.1-fold increase in the immunostaining of Hsp-40, GSK3β and ROCK-II was detected when comparing to SNI, respectively (Fig. 5*C,D*). This data further supports that DRI and SNI elicit differential activation and transport of injury signals to the DRG.

Inhibition of growth cone formation and axonal regeneration is widely described as occurring through activation of the Rho pathway, that signals through its effector ROCK (Mueller et al., 2005). In this context, increased ROCK-II activity in the axoplasm after DRI may certainly contribute to the decreased intrinsic growth capacity of neurons after this type of injury. Consistent with increased ROCK-II activity after DRI, increased phosphorylation of the ROCK-II substrate myosin-light chain was found after DRI (data not shown). In the case of GSK3β, its activation has been linked to axon growth inhibition (Dill et al., 2008; Alabed et al., 2010). GSK3β activity is regulated by phosphorylation; under resting conditions, the kinase is constitutively active following phosphorylation of its

Tyr216 residue and as a response to the activation of the PI3K pathway, GSK3β is inactivated by phosphorylation of Ser9 (Zhou and Snider, 2005). We tried to infer GSK3β activity on DRG by checking its phosphorylation status. Increased levels of both Ser9 and Tyr216 phosphorylation following DRI were detected by Western blot (Fig. 5*A*). To further determine whether increased GSK3β activity was present after DRI, we assessed the levels of pCRMP2, a GSK3β target. Following DRI, p-CRMP-2 was increased supporting a higher GSK3β activity (data not shown). Hsp-40 is a member of the heat shock protein family where in contrast to ROCK-II and GSK3β, limited information is available as to its role in axon growth and regeneration. To elucidate whether Hsp-40 plays a direct role in axon growth, we tested neurite outgrowth *in vitro* following knockdown of Hsp-40 by ShRNA. However, as expected from its function as a stimulator of the activity of the chaperone Hsp-70 (Fan et al., 2003), downregulation of Hsp-40 led to neuronal toxicity (data not shown). This data suggests that increased Hsp-40 following DRI is probably related to neuroprotection and not to a direct effect in axon growth. Supporting this hypothesis, it is known that induction of Hsp protein expression improves motoneuron survival following injury (Kalmar et al., 2002). In summary, upregulation of ROCK-II and GSK3β following DRI might act as a negative injury signaling contributing to the inability of DRG neurons to regenerate robustly.

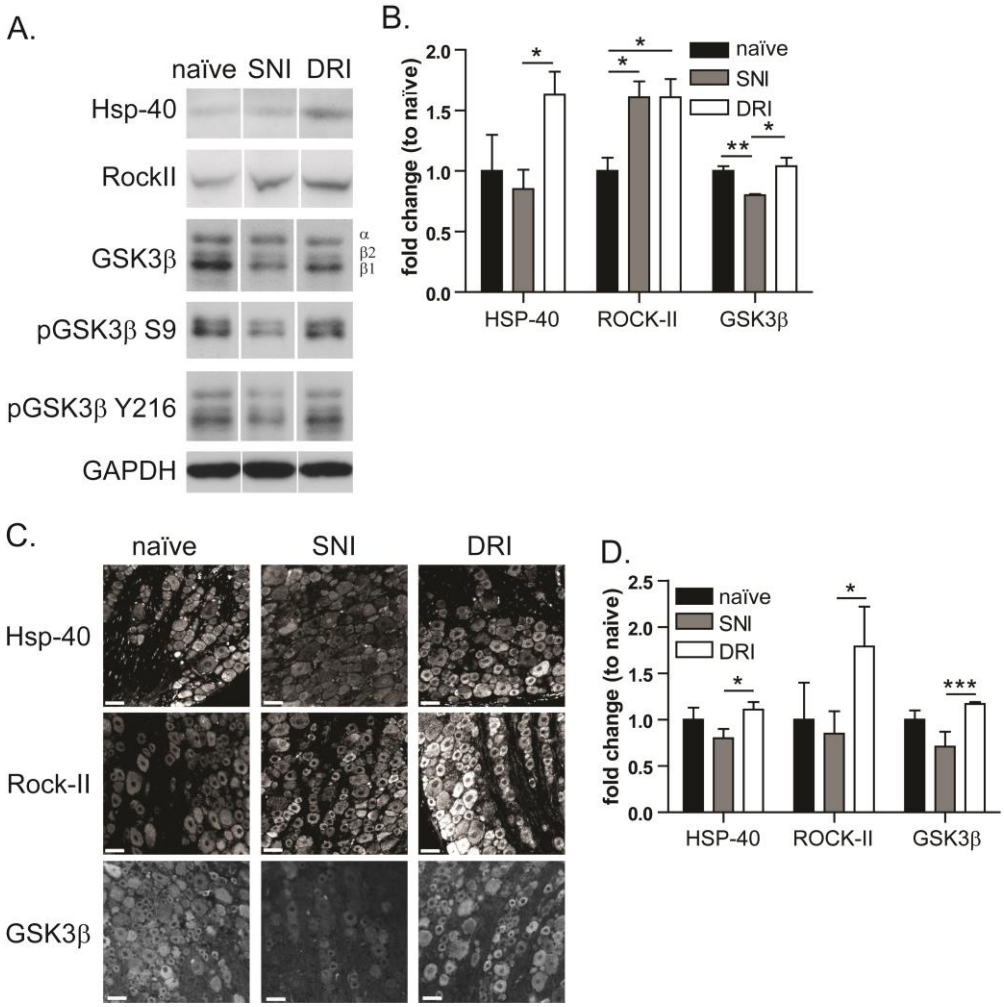

Figure 5. Hsp-40, ROCK-II and GSK3β are increased in the DRG following DRI. ***A***, Western blot for Hsp-40, ROCK-II, GSK3β, pGSK3β S9, pGSK3β Y216 and GAPDH in L4,5 DRG from naïve animals or animals with SNI or DRI. ***B***, Quantification of **A**. ***C***, Immunohistochemistry against Hsp-40, ROCK-II and GSK3β in L4,5 DRG from naïve animals or animals with either SNI or DRI. ***D***, Quantification of **C**. Results represent the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001.

Discussion

CNS injury does not trigger a gain of intrinsic growth capacity, as it fails to induce the expression of RAGs. In the PNS, ERK, JNK and STAT-3 are activated following injury, and their transport to the cell body is important for the regenerative response (Cavalli et al., 2005; Perlson et al., 2006; Ben-Yaakov et al., 2012). Here, we show that after DRI, a simplified model of CNS injury, activation of the intrinsic growth capacity of DRG neurons fails despite that activation of injury signaling occurs. This observation raises intriguing questions on the relevance of ERK, JNK and STAT-3 as master regulators of the regenerative response. Following activation, ERK retrograde transport is dependent on vimentin, and further supporting the importance of this signal, vimentin knockout nerves, lack a regenerative response (Perlson et al., 2005). Nevertheless, this signaling pathway is not present in all DRG neurons, since 40% of DRG neurons do not express vimentin (Perlson et al., 2005). This evidence suggests that ERK alternative pathways may be important to mount the regenerative response which is further supported by our results since when ERK phosphorylation is inhibited, DRG neurons are still able to increase their intrinsic growth capacity. Given the robust response increase in pJNK following injury, it would be interesting to know whether downregulation of JNK signaling is sufficient to impair the regenerative response. In the case of STAT-3, its activity is inhibited by suppressor of cytokine signaling 3 (SOCS3), and further supporting the importance of STAT-3 signaling, axonal regeneration can be improved by SOCS3 deletion (Sun et al., 2011), or by increasing STAT-3 activity (Lang et al., 2013). However, the presence of activated STAT-3 following DRI, when the intrinsic growth capacity is not increased, raises the possibility that other inhibitory pathways may suppress its function. Although ERK, JNK and STAT-3 are the most studied retrograde injury signals, recent analysis of axoplasm following peripheral injury revealed that many other retrograde signals may exist, supporting the robustness and redundancy of the regenerative response to peripheral injury (Michaelevski et al., 2010). Inactivation of PKCα, and activation of ABL, AKT and p38 were identified as signaling pathways that improve axonal regeneration following peripheral injury (Michaelevski et al., 2010). In our antibody microarray, we did not find major differences in PKCα, ABL, AKT and p38, suggesting that regenerative differences between SNI and DRI are probably not due to alterations in these signaling pathways.

In our model, by comparing the axoplasm from nerves with different regeneration abilities, we identified several signals upregulated and downregulated after DRI. From these, we show that ROCK-II, GSK3β and Hsp-40 have an increased transport up to the cell body following DRI. Their presence in the cell body in conditions where there is no gain in the intrinsic regeneration capacity after injury suggests that they may act as axonal growth inhibitors. In fact, both ROCK-II and GSK3β have been widely associated to axonal growth inhibition. Myelin proteins, namely Nogo, MAG and OMgp, are strong inhibitors of axonal growth (Yiu and He, 2006). Following receptor binding, there is activation of the small GTPase RhoA that signals through its effector ROCK. The usage of RhoA/ROCK pathway inhibitors, such as the Rho inhibitor C3 transferase (Dergham et al., 2002) and ROCK inhibitors as Y-27632 (Dergham et al., 2002) and fasudil hydrochloride (Alabed et al., 2006), have been largely used to surpass myelin inhibition and induce axonal regeneration. Further reinforcing the importance of this pathway in signaling axon growth inhibition, ROCK-II dominant negative neurons are able to surpass myelin inhibition (Alabed et al., 2006), and ROCK-II KO mice present increased axonal regeneration (Duffy et al., 2009). The inhibitory effect produced by ROCK is attributed to local alterations in the cytoskeleton at the axon tip (Yiu and He, 2006). We observed that following DRI, increase levels of ROCK-II are also observed at the cell body. Further studies should unravel how ROCK-II transport to the cell body may lead to axon growth inhibition. Besides the RhoA/ROCK pathway, activation of GSK3β has been linked to axonal growth inhibition and GSK3β inhibitors were successfully used to promote axonal growth in the presence of inhibitory substrates *in vitro,* and *in vivo* following spinal cord injury (Dill et al., 2008). GSK3β is able to regulate axonal growth by manipulating microtubule dynamics (Zhou and Snider, 2005). In our model, similarly to ROCK-II, we observed that there is increased GSK3β in the cell body. In fact, a small fraction of GSK3β has been described as having a nuclear localization, with a higher kinase activity (Bijur and Jope, 2003). How this specific distribution of GSK3β impairs axon growth remains elusive. Hsp-40 is a member of the heat shock protein family, which is known to be upregulated under pathologic conditions such as neurodegenerative diseases where it seems to play a protective role (Jana et al., 2000). Following a central injury, neurons may require an increase in protection mechanisms such as the ones provided by heat shock proteins, justifying the increased levels of Hsp-40 following DRI.

The reasons underlying the broad differences in injury signaling response after lesion to the central or to the peripheral branch of a DRG neuron remain elusive. Whether these arise as a consequence of intrinsic differences of both DRG branches and/or have a contribution of their distinct extracellular environments should be further addressed.

In summary, our data shows that DRI and SNI lead to a broad differential activation and transport of signals in the axoplasm, and that the ERK, JNK, STAT-3 and cAMP signaling pathways are probably minor contributors to the changes in the intrinsic axon growth capacity observed in these injury models. Besides, we provide evidence supporting the existence of inhibitory injury signals that include ROCK-II and GSK3β, which are increased in conditions where a pro-regenerative program is not activated and suggest that their retrograde transport following DRI may work as a break to regeneration.

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Chapter III

CNS axons globally increase axonal transport after peripheral conditioning

Abbreviated title: Increased axonal transport after conditioning

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Abstract

Despite the inability of CNS axons to regenerate, an increased regenerative capacity can be elicited following conditioning lesion to the peripheral branch of dorsal root ganglia neurons (DRGs). By *in vivo* radiolabeling of rat DRGs, coupled to mass spectrometry and kinesin immunoprecipitation of spinal cord extracts, we determined that the anterograde transport of cytoskeleton components, metabolic enzymes and axonal regeneration enhancers, was increased in the central branch of DRGs following a peripheral conditioning lesion. Axonal transport of mitochondria was also increased in the central branch of *Thy1-MitoCFP* mice following a peripheral injury. This effect was generalized and included augmented transport of lysosomes, synaptophysin- and APP-carrying vesicles. Changes in axonal transport were only elicited by a peripheral lesion and not by spinal cord injury. In mice, elevated levels of motors and of polyglutamylated and tyrosinated tubulin were present following a peripheral lesion and can explain the increase in axonal transport induced by conditioning. In summary, our work shows that a peripheral injury induces a global increase in axonal transport that is not restricted to the peripheral branch, and that, by extending to the central branch, allows a rapid and sustained support of regenerating central axons.

Introduction

Although in the adult CNS axon regeneration fails, some CNS axons can be prompted to regenerate. For example, the central branch of dorsal root ganglia neurons (DRGs) gains regenerative capacity when the peripheral branch is injured- a paradigm known as conditioning lesion (Neumann and Woolf, 1999). Numerous molecular pathways are regulated by conditioning lesion but none alone is able to fully replicate its effect.

As neurons are highly polarized, many newly synthesized regeneration-associated genes (RAGs), and cytoskeleton proteins need to be transported anterogradely to the axon tip. In fact, the speed of axonal regeneration is similar to the rate of the slow component b (SCb) of anterograde transport, supporting the relevance of transport in sustaining regeneration (Wujek and Lasek, 1983). Anterograde axonal transport is divided into the slow component a (SCa), which transports neurofilaments, tubulin and microtubule-associated proteins, and SCb, which transports cytoplasmic proteins, such as glycolytic enzymes and actin, whereas vesicles and membranous organelles are transported in the fast component (Lasek et al., 1984). The motors of slow and fast components are similar, and the different rates result from intermittent pausing behavior of cargoes (Roy et al., 2007). Retrograde transport is also critical for regeneration, as it participates in injury signaling (Abe and Cavalli, 2008).

Despite the above evidence suggesting a central role of axonal transport during regeneration, the modulation of transport by injury is not well understood. Initial studies suggested that enhanced axonal growth after conditioning lesion was related to increased transport of cytoskeleton components (McQuarrie and Grafstein, 1982; Oblinger and Lasek, 1988; McQuarrie and Jacob, 1991). However, a more detailed interrogation using current approaches is missing. In this work we used the conditioning lesion to further understand whether axonal transport is regulated in conditions of increased axonal regeneration.

Materials and Methods

Surgeries. 8-10 weeks-old male Wistar rats were handled according to European Union rules. For sciatic nerve injury (SNI), the sciatic nerve was transected at the mid-thigh. For spinal cord injury (SCI), a dorsal hemisection was performed at T8. Rats with conditioning were subjected to SNI 1 week prior SCI.

Radiolabeling of DRG neurons. L4,5 DRGs of 8-10 weeks-old naïve rats and of rats with a SNI performed 1 day before (n=6 rats/group), were injected with [^{35}S]-methionine/cysteine (2 µl [22 µCi])/DRG). Six days later, SCI was performed. One week later, spinal cords were collected from L5 up to the injury site, and segmented into 6 fragments. Each fragment was homogenized and counts per minute of 50 µg of protein were measured. Two-dimensional gels of the spinal cord fragment L4,5 were performed and exposed to a phosphor screen for 5 weeks. Radioactive spots were trypsin-digested and identified by MALDI-TOF/TOF.

qRT-PCR. L4-5 DRGs from 8-10 weeks-old naïve rats and rats 1 day or 1 week following SNI were collected. qRT-PCR was performed using iQ supermix (Bio-Rad).

Immunoprecipitation. 8-10 weeks-old Wistar rats, with and without a conditioning SNI (performed 1 week before SCI), were subjected to SCI and 1 week later the spinal cord (T9-T12) was collected (pool of 5 animals/group). Samples were homogenized in PBS containing 0.3% Triton X-100, protease inhibitors (GE Healthcare) and 1mM sodium ortovanadate. For immunoprecipitation, anti-kinesin heavy chain (Millipore) was used. As negative control, mouse IgG were used. The immune complex was collected using protein G magnetic beads (GE Healthcare). For Western blots, antibodies against RhoGDI (1:2,000 Santa Cruz Biotechnology), CRMP-5 (1:3,000, Prof Améli-Moradi, Université Lyon1, France), pan 14-3-3 (1:1,000, Cell Signaling) and PGP9.5 (1:1,000, AbD Serotec) were used.

Lentivirus production. HEK293T cells were transfected with packaging plasmids pPAX and pLP/VSVG (Dr. Relvas, IBMC) and a lentiviral vector (pLKO) coding for puromycin resistance and expressing the shRNA of interest (Sigma-Aldrich). 48 h later viral titration was done by infecting HEK 293T cells.

Neurite outgrowth. DRG from naïve rats were isolated as described (Miranda et al., 2011). One day after plating, cells were infected with lentivirus (5,000 IU/well) for 12-16 h, and 24 h later treated with puromycin (5µg/mL) for 48 h. To confirm

shRNA efficiency, qRT-PCR for RhoGDI, CRMP-5 and PGP9.5 was performed; a reduction of 85%, 60% and 95% was obtained, respectively. For neurite outgrowth, cells were trypsinized, replated for 12 h and βIII-tubulin immunocytochemistry (1:2000, Promega) was done. At least 150 neurons/condition were traced using NeuronJ. For transfection, 200,000 cells were nucleofected (Amaxa 4D) with R18-EGFP or control plasmid (Dr. Fournier, McGill University, Canada). Cells recovered for 48 h, and 12 h after being trypsinized and replated, neurite outgrowth was measured.

Mitochondria transport. The *MitoMouse* line P (mice of either sex expressing a mitochondrial targeting sequence fused to CFP under the neuronal thy1 promoter) (Misgeld et al., 2007) was used. DRG explants from 8-10 weeks-old mice subjected to SNI were analyzed 1 day or 1 week following injury. In additional experiments, *MitoMice* were subjected to SCI and 2 weeks later a SNI was performed; DRG explants were analyzed 1 week later. Naïve animals served as controls. Explants of L4,5 DRGs with attached dorsal roots and peripheral branches were imaged for up to 2 h. Transport of axonal mitochondria was analyzed by confocal microscopy with acquisition at 0.8Hz for 5 min. The number of transported mitochondria was determined as described (Misgeld et al., 2007). The percentage of moving mitochondria was evaluated in 3 different time frames/axon in at least 12 axons/condition. Mitochondria moving in 10 consecutive frames were used to calculate velocities in at least 50 mitochondria/condition. To image DRG explants from animals with SCI, injured axons were labeled with 1% cholera toxin B (B)-Alexa488 (Invitrogen) by performing 3 injections (total volume 7 µl) in the injured dorsal caudal spinal cord, immediately following lesion. Four days later, CTB-Alexa488-positive axons in the branches of L4,5 DRGs were imaged.

Transport in DRG neurons. Neuron cultures from L4,5 DRG of naïve or conditioned mice (DRG collected 1 week following SNI) of either sex were performed. Cells were either infected with baculovirus expressing synaptophysin-GFP (Invitrogen), incubated with 100 nM lysotracker (Invitrogen) or nucleofected with APP-YFP (Dr Kaether, Leibniz Institute for Age Research, Germany). Vesicle velocity was measured as above, and number of moving APP-containing vesicles was determined as the number of vesicles crossing a vertical line in either direction/minute. The effect of rolipram (0.5µM in DMSO) in axonal transport was

tested in DRGs, by analysis of synaptophysin and lysosome transport as described above.

Western blotting. One day or 1 week following SNI, mouse dorsal roots were collected. Naïve animals served as controls. Five animals/group were used. Antibodies against kinesin heavy chain (1:500, Millipore), cytoplasmic dynein (1:250, Millipore), β-actin (1:5,000 Sigma-Aldrich), α-tubulin (1:1,000, Sigma-Aldrich), acetylated tubulin (1:5,000, Sigma-Aldrich), tyrosinated tubulin (1:2,000, Arium) and polyglutamylated tubulin (1:4,000, Adipogen) were used.

Statistical analysis. All values are mean ± SEM. Student's t-test was used.

Results

A conditioning injury increases the expression and anterograde transport of newly synthesized axonal proteins

To test whether a conditioning injury increases the intrinsic ability of DRGs to regenerate by eliciting the synthesis and anterograde transport of RAGs, we revisited radiolabeling assays used to characterize the different components of anterograde transport (McQuarrie and Grafstein, 1982; Wujek and Lasek, 1983; Lasek et al., 1984; Oblinger and Lasek, 1988), but focusing on transport in the central branch, and coupling radiolabeling with mass spectrometry. In rat spinal cords where SNI preceded SCI (conditioning lesion group), increased amounts of radiolabeled proteins were consistently found (Fig. 1*A*), suggesting increased synthesis and/or anterograde transport. This increase was confirmed by 2D gel analysis of the L4,5 spinal cord fragment, where a generalized 2 to 3-fold increase in radiolabeled protein content was found in spinal cords of animals with conditioning lesion, when compared to animals where only SCI was performed (Fig. 1*B*). In naïve spinal cords, the radiolabeled protein content was similar to that of spinal cords from animals with SCI, whereas that of conditioned animals was undistinguishable from animals with SNI only (not shown). This data shows that increased protein synthesis by DRGs and/or transport to the central branch are elicited by the priming peripheral lesion and that this increase is not recapitulated by a central lesion. The two most abundant proteins identified were cytoskeletal components, tubulin and actin (Fig. 1*B*, spots 2 and 5, respectively). Most of the proteins corresponded to metabolic enzymes (spots 1- NADH dehydrogenase, NDUS1; 4- catalase; 6- creatinine kinase; 8- glutamine synthase; 11 and 12- acetyl-CoA acetyltransferase, acyl coenzyme A thioester hydrolase and prolyl isomerase; 14 and 15- malate dehydrogenase), amongst which several glycolytic enzymes were found (spots 10- fructose-bisphosphate aldolase C; 16 and 17- glyceraldehyde-3-phosphate dehydrogenase; 9- phosphoglycerate kinase 1; 7- α-enolase; spots 3 and 4-pyruvate kinase isozymes) (Fig. 1*B*). In addition, others included heat shock protein 70 (HSP-70- spot 1), dihydropyrimidinase-related protein 5 (CRMP-5- spot 4), G protein subunit 2 (GNB2- spot 13), annexins 2 and 5 (Anxa2 and Anxa 5- spots 16 and 18), sirtuin2 (Sirt2- spot 16), 14-3-3 proteins (isoforms ζ, γ, θ, ζ/δ, η, β/α and ε- spot 19), Rho GDP-dissociation inhibitor 1 (RhoGDI- spot 20) and the neuron-specific ubiquitin carboxyl-terminal hydrolase 1 (PGP9.5- spot 20) (Fig. 1*B*). Of note, as our analysis was performed

following radioactive labelling of DRGs, it was restricted to proteins contained in sensory axons in the spinal cord.

To determine whether the increase in radiolabeled proteins could arise from increased gene expression in DRG, we evaluated the expression of selected proteins identified by 2D gel analysis, namely the cytoskeleton genes βIII-tubulin (tubulin) and β-actin (actin), the glycolytic enzyme GAPDH and the genes coding for CRMP-5, 14-3-3 (ζ isoform), RhoGDI, PGP9.5, HSP-70, GNB2, Anxa2, Sirt2, Anxa5 and NDUS1. Most of the proteins had an increased expression 1 day after SNI, which in some cases was sustained at a later time point (1 week after SNI). Interestingly however, others, including tubulin, actin and anxa5 had unchanged expression following injury (Fig. 1*C*). To test whether proteins identified by 2D gel analysis could also show increased anterograde transport in central axons from conditioned neurons, we performed kinesin-1 immunoprecipitation. In this experimental setup, all the axons of the spinal cord were analyzed and not just sensory axons. In spinal cords of conditioned animals, on average a 2-fold increase in the amounts of CRMP-5, 14-3-3, RhoGDI and PGP9.5 that co-immunoprecipitated with kinesin-1 was found (Fig. 1*D*). This suggests that an increased association with the transport machinery occurs.

Although metabolic enzymes and cytoskeleton components are obvious candidates to be transported to the injury site, many of the remaining proteins identified have not been related to axonal regeneration. Thus, to determine their relevance, the expression or activity of some of these proteins was inhibited in DRG neurons. We observed that neurite outgrowth was inhibited following shRNA-mediated knock-down of RhoGDI and by blocking the 14-3-3 proteins by transfection with R18, a pan 14-3-3 inhibitor (Kent et al., 2010), while the knock down of PGP9.5 and CRMP-5 showed no significant effect (Fig. 1*E*). This suggests that at least for some of the proteins identified by 2D gel analysis, including RhoGDI and 14-3-3, synthesis in the DRG and anterograde transport through the spinal cord may contribute to the increased regenerative capacity induced by conditioning injury.

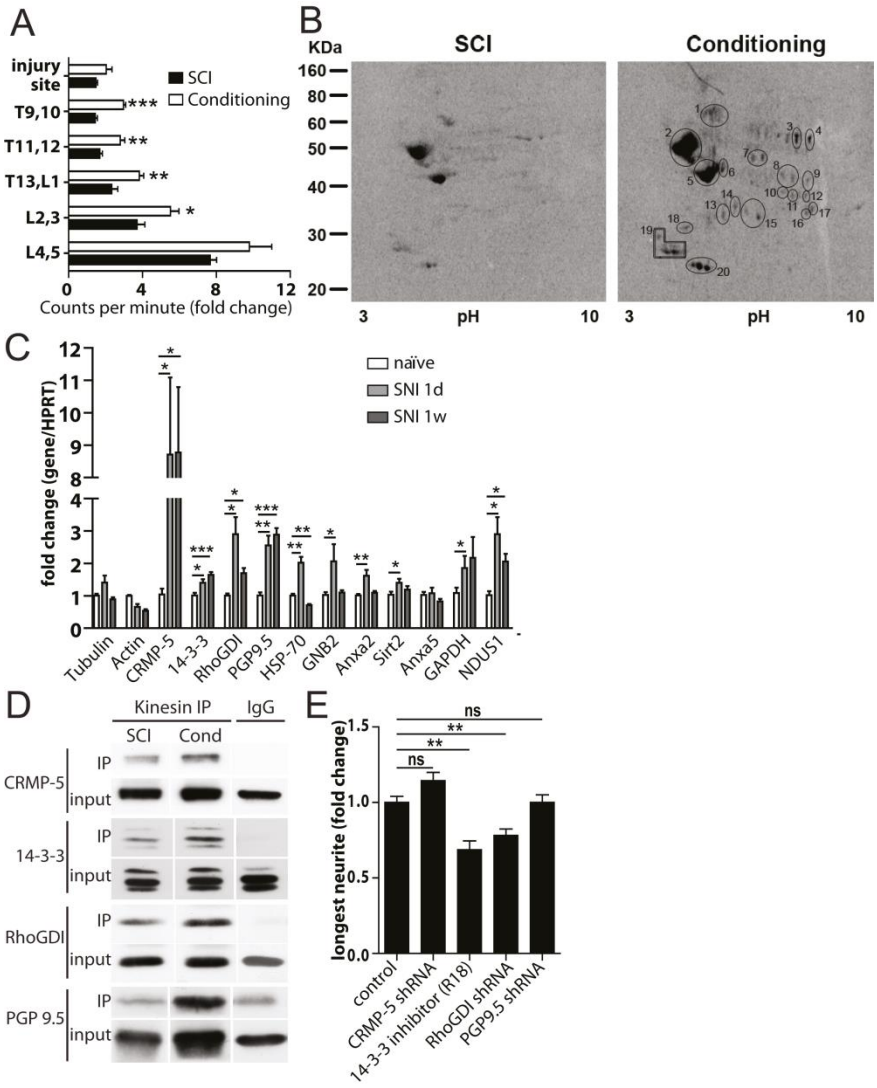

Figure 1. Conditioning lesion increases the synthesis and transport of proteins to the central branch of DRGs. ***A***, Counts per minute in spinal cord fragments of rats with SCI or SCI preceded by SNI (conditioning). ***B***, Representative 2D gels of the L4,5 spinal cord fragment of rats with SCI or conditioning. ***C***, qPCR of DRGs from naïve rats, and from DRGs collected from rats 1 day or 1 week following SNI (SNI 1d and SNI 1w, respectively); n=4 naïve, n=5 SNI 1d and n=10 SNI 1w. ***D***, Representative Western blots of CRMP-5, 14-3-3, RhoGDI and PGP9.5 following kinesin immunoprecipitation of spinal cords of animals with SCI or conditioning. ***E***, Neurite outgrowth of DRGs following transfection with R18, or infection with lentivirus expressing shRNA to CRMP-5, RhoGDI or PGP9.5 (n=3). *p<0.05, **p<0.01 and ***p<0.001.

Transport of mitochondria in the central branch of DRGs is increased following peripheral injury

To determine whether axonal transport of organelles was also affected by a conditioning lesion, we used explants of DRGs from *MitoMice* (Misgeld et al.,

2007) and imaged mitochondrial movements in the attached nerves. A peripheral injury increased the number of transported mitochondria, not only in the peripheral branch (Fig. 2*A*-*C*), but also in the central branch (Fig. 2*A*-*C*). This increase was found both in the anterograde (Fig. 2*B*) and retrograde (Fig. 2*C*) direction. A 1.5-fold increase in anterograde transport was already observed at 1 day following SNI and was sustained 1 week following injury (Fig. 2*B*). In the case of retrograde transport, an increase was only detected 1 week following injury (Fig. 2*C*). At this time point, a conditioning injury also increased the percentage of moving mitochondria both in the peripheral and in the central branch (Fig. 2*D*). Although the conditioning injury led to an increased number of transported mitochondria in the central branch, no increase in the speed of transport was observed (Fig. 2 *E*,*F*).

To evaluate if an injury to the central branch triggered a similar effect in axonal transport, we injected fluorophore-conjugated CTB in the spinal cord immediately following SCI in *MitoMice*, which allowed the identification of axons affected by SCI (CTB-Alexa488-positive; Fig. 2*G*). Injury to central axons failed to produce the same effect as the conditioning injury, since increased transport in the peripheral branch was not observed (Fig. 2*A*,*B*), and a 2.3-fold decrease in the percentage of motile mitochondria (Fig. 2*D*), relatively to naïve animals was found. In the central branch, SCI did not produce changes in mitochondria transport (Fig. 2*B*,*C*,*D*).

Peripheral injury performed after a central injury still increases the regenerative capacity of DRGs (Ylera et al., 2009). In *MitoMice* where SNI was done 2 weeks after SCI, transport was increased similarly to animals with SNI alone. An increased number of moving mitochondria (Fig. 2*A,B,C*) and an increased percentage of motile mitochondria (Fig. 2*D*) were observed. Overall, only a peripheral injury (even if performed after central injury) elicits increased transport of axonal mitochondria.

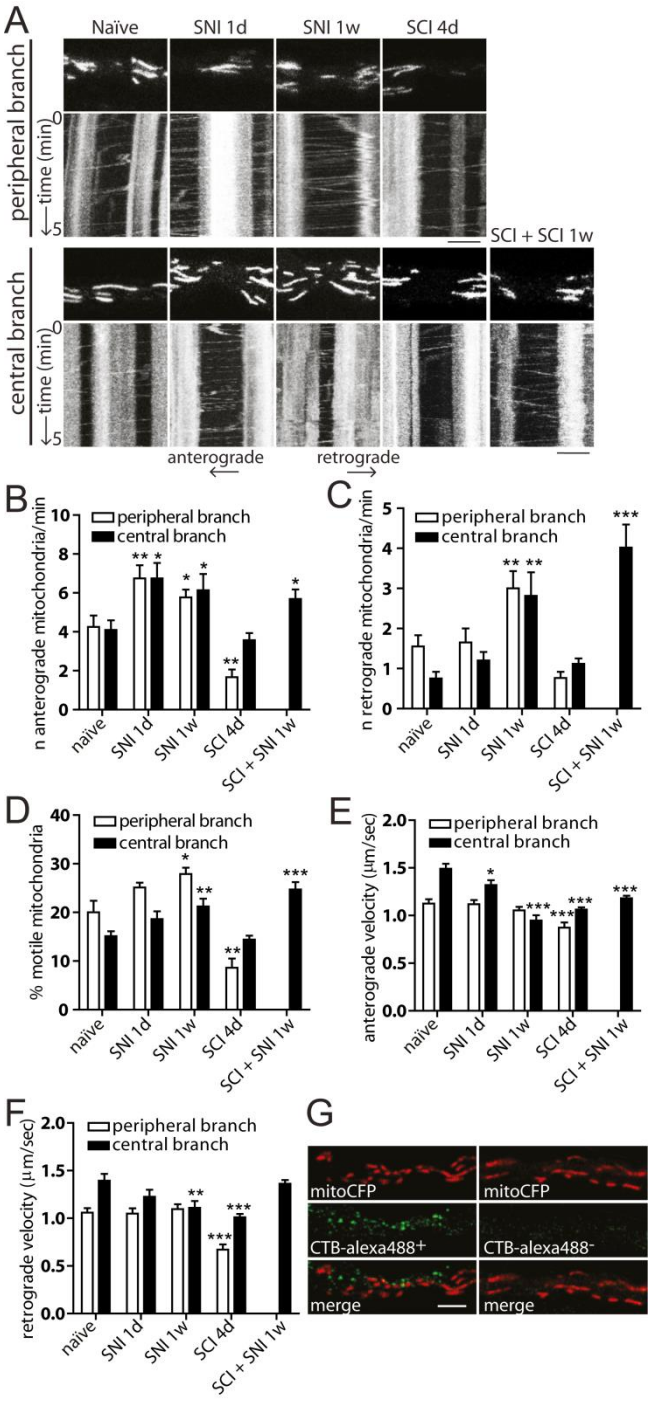

Figure 2. A conditioning injury increases the axonal transport of mitochondria. ***A***, Kymographs of DRG explants from naïve *MitoMice*, *MitoMice* where SNI was performed 1 day or 1 week before (SNI 1d and SNI 1w, respectively), *MitoMice* where SCI was performed 4 days before (SCI 4d) or *MitoMice* where SNI was performed 2 weeks after SCI and analysis was done 1 week later (SCI+SNI 1w; only the central branch was imaged). Upper panel, peripheral branch; Lower panel, central branch. Large vertical bands correspond to stationary mitochondria and fine diagonal lines are single motile mitochondria. (***B-F***) Number of ***B***, anterogradely and ***C***, retrogradely moving mitochondria, ***D***, percentage of moving mitochondria, and speed of ***E***, anterogradely and ***F***, retrogradely moving mitochondria in the peripheral and central branches of DRG explants from naïve and injured *MitoMice* (SNI 1d, SNI 1w, SCI 4d and SCI + SNI 1w). ***G***, Representative micrographs of peripheral

DRG axons of *MitoMice* injected with CTB-Alexa488 in the SCI site, 4 days before. CTB-Alexa488 positive (left) and negative (right) axons are shown. Scale bar: 5µm.

In DRGs, a conditioning injury leads to increased transport of lysosomes, and of synaptophysin- and APP-containing vesicles

To evaluate how broad the alterations in axonal transport induced by conditioning lesion would be, we assessed axonal transport of lysosomes, synaptophysin and APP in DRGs from either naïve or conditioned animals. In DRGs labeled with lysotracker, whereas a conditioning injury did not alter the percentage of moving lysosomes (naïve: 40.1±3.0%, conditioning: 40.3±3.4%), the speed of transport was 1.6-fold increased specifically in the anterograde direction (Fig. 3*A,B*). In DRGs transduced with synaptophysin, similar changes were observed. Whereas no differences were detected in the percentage of moving synaptophysin-containing vesicles (naïve: 63.9±3.5%, conditioning: 56.0±4.0%), a 1.5-fold increase in the speed of anterograde transport of synaptophysin was found in conditioned DRGs (Fig. 3*C*,*D*). Following APP-YFP transfection, a 2.2-fold increase in the number of anterogradely transported APP-containing vesicles (Fig. 3*E,G*), and a 1.3-fold increase in speed of transport in both directions, were detected (Fig. 3*F*). In summary, a conditioning injury leads to broad alterations in axonal transport, affecting the transport of proteins, organelles and vesicles, particularly in the anterograde direction.

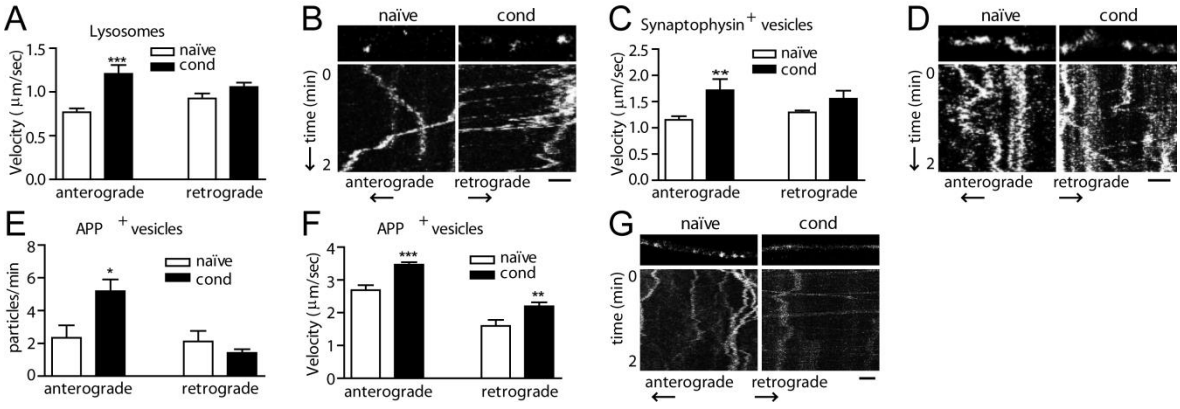

Figure 3. Conditioning injury increases the transport of lysosomes, and of synaptophysin- and APP-containing vesicles. ***A***, Velocity of anterogradely and retrogradely moving lysosomes in naïve and conditioned DRGs (at least 48 lysosomes/condition). ***B***, Kymographs of DRGs from naïve or conditioned (cond) animals with lysosomes labeled with lysotracker. Scale bar: 2µm. ***C***, Velocity of anterogradely and retrogradely moving synaptophysin-positive vesicles in naïve and conditioned DRGs (at least 44 synaptophysin-positive vesicles/condition). ***D***, Kymographs of DRGs from naïve or

conditioned (cond) animals transduced with synaptophysin-GFP. Scale bar: 2µm. ***E***, Number of anterogradely and retrogradely moving APP-positive vesicles in naïve and conditioned DRGs. ***F***, Velocity of anterogradely and retrogradely moving APP-positive vesicles in naïve and conditioned DRGs (at least 42 APP-positive vesicles/condition). ***G***, Kymographs of DRGs from naïve or conditioned animals transfected with APP-YFP. Scale bar: 2µm. *p<0.05, **p<0.01 and ***p<0.001.

Increased levels of molecular motors and of polyglutamylated tubulin can underlie the early increase in axonal transport elicited by a conditioning lesion

To understand the mechanism underlying the increased axonal transport in the central branch of conditioned DRGs, we analyzed whether conditioning would elicit alterations previously associated to the control of axonal transport, namely in molecular motor availability and suitable microtubule tracks. In DRGs, SNI was accompanied by 1.5- and 1.8-fold increased RNA levels of kinesin-1B and dynein-1, respectively, as early as 1 day following SNI (Fig. 4*A*). In the case of kinesin-1B, this increase was not sustained, but dynein-1 was still increased at 1 week following SNI (Fig. 4*A*). At the protein level, only kinesin-1 was significantly increased in the dorsal root 1 day following SNI but not 1 week following SNI (Fig. 4*B*). The anterograde transport of cargoes can be regulated by phosphorylation of kinesin light chain 1 (KLC1), as increased phosphorylation leads to disruption of cargoes from the transport complex (Morfini et al., 2004). However, decreased levels of dephosphorylated KLC1 were present in dorsal roots 1 week after SNI (Fig. 4*C*), failing to explain increased anterograde transport.

Since tubulin modifications can alter the binding affinity to microtubules, we tested whether changes in tubulin in the dorsal root could explain the conditioning-induced transport alterations. Tyrosination of tubulin is a marker of dynamic microtubules and can inhibit kinesin-1 binding to microtubules (Dunn et al., 2008). Interestingly, increased levels of tyrosinated tubulin were found 1 day and 1 week following conditioning injury (Fig. 4*D*,*E*), suggesting a more dynamic state of dorsal root microtubules. Tubulin acetylation has been associated with increased anterograde transport given improved kinesin-1 binding to microtubules (Reed et al., 2006). However, no alterations in tubulin acetylation were found (Fig. 4*D,F*). As kinesin-1 also binds more effectively to polyglutamylated tubulin (Ikegami et al., 2007), this modification was subsequently evaluated. Indeed, 1 day following conditioning injury, polyglutamylated tubulin was increased in dorsal roots (Fig. 4*D,G*). However, this

alteration was not sustained (Fig. 4*G*). In summary, increased levels of kinesin and polyglutamylated tubulin can underlie, at least partially, the early increase in anterograde axonal transport elicited by conditioning lesion.

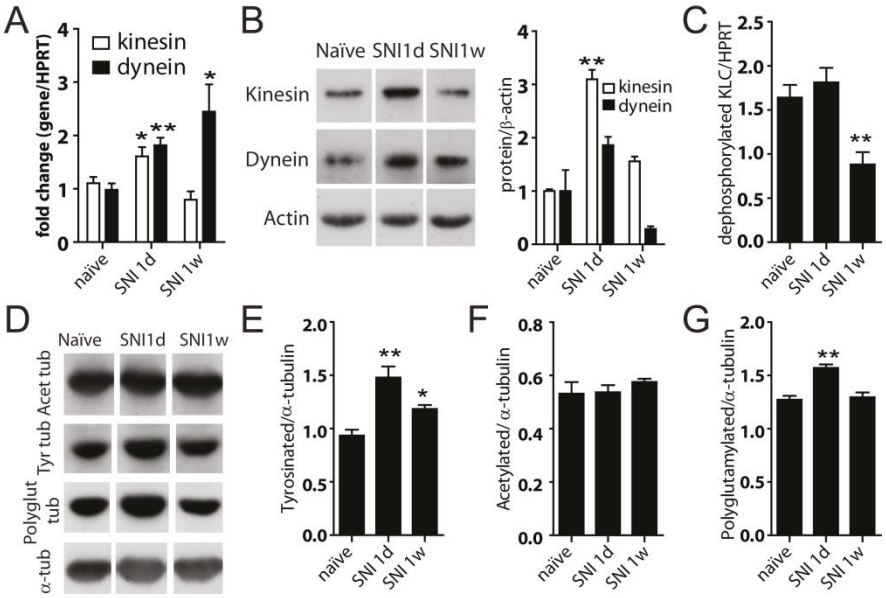

Figure 4. Increased levels of molecular motors and of polyglutamylated and tyrosinated tubulin are elicited following conditioning lesion. ***A***, qPCR of kinesin and dynein expression in DRG from either naïve rats or collected from rats 1 day or 1 week following SNI (SNI 1d and SNI 1w, respectively; n=7 naïve, n=4 SNI 1d and n=7 SNI 1w). ***B***, Representative Western blots and quantification of kinesin and dynein in L4,5 dorsal roots from either naïve rats or collected from SNI 1d and SNI 1w; n=4/group. ***C***, Quantification of Western blots of dephosphorylated KLC in L4,5 dorsal roots from either naïve rats or collected from SNI 1d and SNI 1w; n=5/group. ***D***, Representative Western blot analysis of tubulin in L4,5 dorsal roots from either naïve rats or collected from SNI 1d and SNI 1w; n=5/group. ***E***, ***F*** and ***G*** Quantification of ***D***. *p<0.05 and **p<0.01.

Discussion

We show that besides broad changes in expression, a peripheral injury induces a global increase in axonal transport. Consequently, once a central injury occurs, any regenerative effort of the central branch can be rapidly supported. While increased cAMP is a central mediator of the conditioning effect (Qiu et al., 2002), cAMP analogs fail to reproduce its full effect (Blesch et al., 2012). Interestingly, cAMP is not able to increase axonal transport (Han et al., 2004). Besides, rolipram, a phosphodiesterase inhibitor used to increase cAMP and promote CNS regeneration (Nikulina et al., 2004), did not alter transport in DRGs (not shown).

We identified several glycolytic enzymes with increased anterograde transport in central axons following conditioning lesion. Interestingly, glycolysis is a central source of energy for vesicle transport (Zala et al., 2013). Besides, transport of cytoplasmic proteins in SCb occurs through intermittent association to membrane-bound vesicles (Tang et al., 2012). Thus, the higher abundance of glycolytic enzymes, together with the increased transport of vesicles following conditioning lesion, may explain the increased transport of axonal proteins here identified.

Our approach allowed the identification of transported putative regeneration enhancers, such as 14-3-3 and RhoGDI. Supporting its relevance during axonal growth, 14-3-3 regulates axon guidance during development (Kent et al., 2010). Similarly, RhoGDI may be critical to regenerating axons as it inhibits RhoA, a central mediator of growth cone collapse (Sasaki and Takai, 1998). Surely, other proteins identified remain as interesting candidates to evaluate during axonal regeneration.

Molecular motors and polyglutamylated tubulin were increased in the dorsal root shortly after a peripheral lesion. As these effects were transient, the sustained increase of axonal transport at later time points remains to be explained. After conditioning lesion, the levels of tyrosinated, likely dynamic microtubules, were persistently increased. A precise control of microtubule dynamics is needed to optimize axon regeneration. Indeed, PNS neurons require decreased microtubule stability in the growth cone to regenerate (Cho and Cavalli, 2012). Hence, persistent alterations in microtubule stability might explain some of the effects observed.

Our work stressed that understanding the intersection between injury-induced signaling and axonal transport is an important aspect to consider. In summary, we propose that a broad increase in axonal transport is a major player during axon regeneration.

Acknowledgments

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Conflicts of interest: The authors declare no competing financial interests.

General conclusion and future perspectives

SCI is a severe condition that usually results in loss of sensitive and motor function, as well as autonomic functions, culminating in lifetime medical care, as the available treatments are limited. As such, the development of novel therapies that may lead to improvements in the patient quality of life are needed. For that, our basic knowledge on both extrinsic and intrinsic mechanisms controlling axon growth and regeneration need to be expanded.

Initially, the limited regeneration of CNS axons was attributed mainly to the highly inhibitory environment formed following SCI. Upon injury, the glial scar functions as a physical barrier, but also as a biological barrier, since it contains numerous axonal regeneration inhibitors, namely myelin proteins and CSPGs. We were able to identify novel axonal regeneration inhibitors present in the injury milieu. Specifically we determined that besides myelin proteins, myelin lipids are able to inhibit axonal growth, particularly cholesterol and sphingomyelin. The use of 2-hydroxypropyl-β-cyclodextrin, a drug capable of sequestering cholesterol and sphingomyelin, *in vivo*, allowed increased axonal regeneration of dorsal column fibers following spinal cord hemisection. It will be interesting to determine now whether 2-hydroxypropyl-β-cyclodextrin is able to promote axonal regeneration of other axonal tracts and to produce functional recovery.

Although several studies have shown improved axonal regeneration by blocking inhibitors present in the glial scar, such results are still shy from a major effect that could be translated to clinical trials. Recently, modifying the intrinsic ability of CNS neurons emerged has an interesting target to promote regeneration. While PNS neurons are able to mount a strong regenerative response following injury, CNS neurons usually fail to produce such response. A key aspect of the ability of PNS neurons to respond to injury is the generation of injury signals upon lesion. Whether these signals are present following a CNS injury is not known. Here, we used the dorsal root injury as a model of CNS injury, since it produces only a mild response to injury and does not result in axon regeneration. Surprisingly, ERK, JNK and STAT-3, the best described injury signals in the PNS, were also activated and retrogradely transported following dorsal root injury. To identify possible differences in the injury signaling mechanism following peripheral and dorsal

root injury, we used an antibody microarray to analyze the axoplasm content upon each lesion. We were able to determine that a dorsal root injury elicits the differential activation of signaling molecules. From these, ROCK-II and Hsp-40 were increased following dorsal root injury whereas GSK3β was decreased after sciatic nerve injury. ROCK-II and GSK3β are known inhibitors of axonal regeneration. Our results, especially for ROCK-II, suggest that following dorsal root injury besides transport of positive injury signals, there is also the transport of signals that may repress regeneration. Hsp-40 has been associated with protection in neurodegenerative diseases. Its knock down in neurons showed signs of toxicity, suggesting that the increase in Hsp-40 following dorsal root injury is probably not a direct player in the regenerative process but instead, participates in protection mechanisms.

The conditioning injury model has been extensively used to dissect the increase in the intrinsic ability triggered by a peripheral injury. By *in vivo* radiolabeling we identified newly synthesized proteins that are anterogradely transported upon conditioning injury. From these, several potential novel RAGs were identified, from which we were able to validate RhoGDI and 14-3-3 as playing a role in neurite outgrowth. There are certainly other candidates identified in our screening that are worth studying in the context of axon growth and regeneration.

Furthermore, our data demonstrated that a conditioning lesion elicited the transport of newly synthesized proteins, organelles and synaptic vesicles. These transport alterations were independent of motor levels, however the changes observed in microtubule post-translation modifications, such as tyrosination and polyglutamylation could at least in part explain the increased axonal transport. At this stage, the molecular mechanisms controlling the increased transport should be further dissected. Although the levels of the motors are similar, following injury is there an increase in the percentage of motile motors? Is the increased transport due to higher run lengths and decreased pauses among run lengths? Does tyrosination and polyglutamylation increase motors binding and transport? Answering these questions could help to further understand the mechanisms that participate in improving axonal transport. Besides, whether increased axonal transport is a general feature of conditions where increased axonal regeneration occurs, as is the case for instance in PTEN and SOCS-3 KO mice, should be further investigated. Combined, this knowledge may allow the future targeting of the

transport machinery as a means of promoting axonal regeneration. Also, it would improve treatment of several neurodegenerative diseases where defficient axonal transport is present.

In summary, we were able to identify novel extrinsic cues that can impair axonal growth such as cholesterol and sphingomyelin, but also novel mechanisms that can contribute to increase axonal regeneration following conditioning injury, namely a robust increase in axonal transport of proteins, mitochondria and vesicles.

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